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Contents

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EDITORIAL

Terlipressin and hepatorenal syndrome: What is important for nephrologists and hepatologists

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Abstract

Hepatorenal syndrome (HRS) is a reversible form of functional renal failure that occurs with advanced hepatic cirrhosis and liver failure. Despite mounting research in HRS, its etiology and medical therapy has not been resolved. HRS encompasses 2 distinct types. Type 1 is characterized by the rapid development of renal failure that occurs within 2 wk and involves a doubling of initial serum creatinine. Type 2 has a more insidious onset and is often associated with ascites. Animal studies have shown that both forms, in particular type 1 HRS, are often precipitated by bacterial infections and circulatory changes. The prognosis for HRS remains very poor. Type 1 and 2 both have an expected survival time of 2 wk and 6 mo, respectively. Progression of liver cirrhosis and the resultant portal hypertension leads to the pooling of blood in the splanchnic vascular bed. The ensuing hyperdynamic circulation causes an ineffective circulatory volume which subsequently activates neurohormonal systems. Primarily the sympathetic nervous system and the renin angiotensin system are activated, which, in the early stages of HRS, maintain adequate circulation. Both advanced cirrhosis and prolonged activation of neurohormonal mechanisms result in fatal complications. Locally produced nitric oxide may have the potential to induce a deleterious vasodilatory effect on the splanchnic circulation. Currently medical therapy is aimed at reducing splanchnic vasodilation to resolve the ineffective circulation and maintain good renal perfusion pressure. Terlipressin, a vasopressin analogue, has shown potential benefit in the treatment of HRS. It prolongs both survival time and has the ability to reverse HRS in the majority of patients. In this review we aim to focus on the pathogenesis of HRS and its treatment with terlipressin *vs* other drugs.

Key words: Heptorenal syndrome; Terlipressin; Kidney; Liver

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INTRODUCTION

Many studies have been carried out on hepatorenal syndrome (HRS); the pathophysiology and its management

however have not been completely resolved. HRS is a reversible form of functional renal failure that occurs predominantly with advanced liver disease arising from hepatic cirrhosis or severe liver injury from any condition such as severe alcoholic hepatitis or metastatic tumors[1]. The important features of HRS are characterized by peripheral vasodilation with subsequent profound intrarenal vasoconstriction, leading to decreased glomerular filtration rate (GFR)[2,3].

Currently, HRS encompasses 2 distinct types. Type 1 HRS often manifests itself rapidly; without appropriate treatment the mean survival time is approximately 2 wk[4]. The distinguishing feature of type 1 HRS is rapid progressive renal failure that occurs within 2 wk and is associated with doubling of baseline serum creatinine or a 50% reduction in creatinine clearance[5]. In more than 70% of cases there is an identifiable trigger for type 1 HRS[6-9]. A large number of studies have shown that type 1 HRS can be precipitated by preceding spontaneous bacterial peritonitis infections, gastrointestinal bleeding and large-volume abdominal paracentesis without albumin replacement[6,8]. Furthermore, type 2 HRS has a gradual onset with a steady decline in renal function. Interestingly, the hallmark for type 2 HRS is refractory ascites and often has no precipitating factors[4]. The survival time is better in type 2 HRS at approximately 6 mo[1,5]. Some would consider injudicious use of diuretics as a precipitating factor.

Importantly, the core feature of pathogenesis of HRS is peripheral arterial vasodilation, in particular in the splanchnic vasculature[10]. This develops with advanced liver cirrhosis, which causes increased resistance to blood flow with high portal pressure. In turn, to ease the pressure within the hepatic portal system, locally acting vasoactive substances are released that cause vasodilation of the splanchnic vasculature[10]. The overall resultant effect is circulatory dysfunction arising from a depleted intravascular volume that ultimately leads to poor renal perfusion and activation of compensatory mechanisms (renin angiotensin aldosterone system, sympathetic nervous system and vasopressin). These compensatory mechanisms with time become detrimental and result in sustained severe intrarenal arterial vasoconstriction with progressive physiological renal failure[2]. The pooling of blood in the splanchnic vascular bed with the associated hypoperfusion of the kidneys and the ensuing intrarenal arterial vasoconstriction forms the basis for the development of HRS.

HRS has very poor prognosis with spontaneous recovery being unlikely[2]. Treatment of HRS can be divided into medical and surgical, the latter being more beneficial. Current treatment modalities are used as a bridge to surgical intervention (liver transplant), although most patients do not survive long enough to receive a liver transplant[2]. Pharmacotherapy is the initial treatment which buys time for a liver transplant but unfortunately there is no universally agreed first-line therapy. There are a number of pharmacological agents that have been investigated in the management of HRS and thus far most drugs aim to reverse the peripheral and splanchnic vasodilation. Usually treatment is a combined therapy of vasoconstrictors with albumin to augment their efficacy[11].

Vasoconstrictive drugs such as vasopressin analogues (ornipressin, terlipressin), octreotide and noradrenaline have been used in attempts to reduce the pooling of blood in the splanchnic vasculature and the peripheral arterial vasodilation[12]. A few studies have investigated ornipressin combined with albumin or dopamine and they have been shown to reverse HRS[13-15]. Globally the use of ornipressin has been abandoned in HRS because of the high risk of an adverse event, in particular ischemic events. Other studies have shown that with the use of potent vasoconstrictors such as ornipressin, the result can be ischemic mesenteric mucosa, myocardial ischemia, and associated ventricular arrhythmias[16]. A safe alternative treatment is terlipressin (vasopressin analogue) which so far has shown promising results.

Several studies have confirmed that terlipressin combined with albumin achieves acceptable GFR and it almost normalizes the plasma creatinine levels in 42% to 77% of cases[17-20]. The aim of the current review is to evaluate the recent developments made in the pathogenesis of HRS and the role of terlipressin, including its possible mechanism of it action.

PATHOGENSIS OF HRS

The etiopathogenesis of HRS has not been fully resolved and there are possible theories to explain it at a cellular and molecular level. The defining feature of HRS is profound vasoconstriction of the renal vasculature due to inadequate blood flow to the kidneys[21-24]. The culmination of several factors leads to the development of HRS: (1) portal hypertension (PHT); (2) altered peripheral blood circulation; (3) activation of the sympathetic nervous system; and (4) the release of chemical mediators.

PHT AND NITRIC OXIDE

Over time, liver cirrhosis leads to structural changes at both a microscopic and macroscopic level within the hepatocytes. The pressure within the hepatic microcirculation becomes raised and the so-called sinusoidal PHT occurs[25]. Furthermore, this is complemented by the ongoing changes taking place within the myofibroblasts, stellate cells and portal venules which all contribute towards the development of increased resistance to portal blood flow[26,27]. Most research on animals has suggested 2 possible theories to explain the development of PHT: (1) "forward theory" - this theory puts forward that PHT arises as a direct consequence of increased resistance to portal inflow; and (2) "backward theory" which proposes that PHT occurs due to high portal blood inflow because of a hyperdynamic circulation[28,29]. Furthermore, it is insinuated that this abnormally high portal blood inflow sustains the PHT[30].

In spite of the etiology of PHT, some of the portal venous blood gets redirected *via* collaterals vessels and this is partially to take pressure off the portal system[31]. Gradu-

ally, with persistent PHT, local and systemic changes occur; neurohormonal systems are activated and locally produced vasoactive substances such as nitric oxide are released[32]. Other locally acting vasodilatory substances released include carbon monoxide and prostacyclin[33]. Nitric oxide however is widely believed to be one of the main culprits for initiating the splanchnic arterial vasodilation[34].

In animal models nitric oxide has been shown to play an important role in vascular tone and splanchnic vasodilation[35,36]. In other animal studies, it is postulated that the production of nitric oxide may be related to bacteria stimulating macrophages which in turn induce nitric oxide synthase (NOS)[37-39]. NOS is an enzyme that forms nitric oxide from L-arginine, which is found throughout the body in numerous different types of cells. In addition, NOS has been shown to have 3 isoforms which are NOS I - neuronal NOS (nNOS), NOS Ⅱ - inducible NOS (iNOS) and NOS Ⅲ - endothelial NOS (eNOS)[40-42].

Isoform nNOS is primarily found in the central nervous system and it has been shown to have a key role in controlling blood pressure. Several studies on rats have demonstrated that by inhibiting this isoenzyme it generates increased sympathetic activity with ensuing tachycardia, and hypertension[43-45]. Conversely, iNOS is present in humans in several tissues including hepatocytes and alveolar macrophages; its release is induced by several cytokines including interleukin 1, interferon γ, tumor necrosis factor and lipopolysaccharides[46,47]. Finally eNOS, as the name suggests, is predominantly found in endothelial cells in humans in both arterial and venous vessels[48,49]. In the literature, eNOS has been shown to be involved in the peripheral arterial vasodilation that occurs in HRS and there are raised levels of eNOS in the circulation[48]. Overall eNOS has an important role in maintaining sympathetic vascular tone and can be synthesized within the endothelium in response to stimuli.

HYPERKINETIC CIRCULATION AND COMPENSATORY MECHANISM

The hemodynamic changes that develop in cirrhosis in the splanchnic circulation have been studied extensively, and only slow progress has been made in determining its pathophysiology. A number of plausible theories have been postulated in the last 2 decades based on both *in vitro* and *in vivo* studies. Hyperdyanamic circulation is a phenomena that happens over a period of time as a direct consequence of long standing PHT (Figure 1)[50]. The hallmarks of this circulatory dysfunction are tachycardia, increased cardiac output and abnormally low peripheral vascular resistance with decreased arterial blood pressure[31].

Hyperkinetic circulation develops in several steps: (1) splanchnic and peripheral vasodilation; (2) an increase in total blood volume with inadequate circulating volume; (3) increased cardiac output; and (4) the activation of a compensatory mechanism. Initially there is pooling of blood in the splanchnic vasculature due to PHT and this causes decreased circulatory volume.

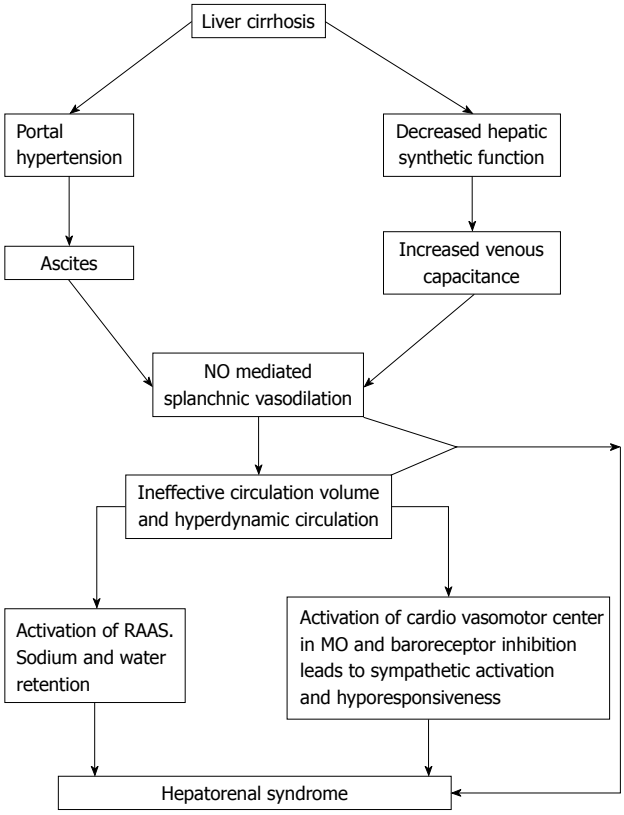

Figure 1 Flow chart showing the vicious cycle that develops with decompensated liver cirrhosis and the serious of events that lead to hepatorenal syndrome. NO: Nitric oxide; RAAS: Renin angiotensinogen aldosterone system; MO: Medulla oblongata.

One of the earliest indicators of a hyperdynamic circulation is the redistribution of blood volume into the splanchnic circulation. This event has been demonstrated in Doppler ultrasonography studies in which patients with cirrhosis had remarkably high splanchnic blood flow when compared to normal subjects[51]. The development of increased blood flow to splanchnic circulation and the pooling of blood produce a decreased circulating volume that triggers neurohormonal responses.

SYMPATHETIC NERVOUS SYSTEM, CARDIAC OUTPUT AND THE BARORECEPTORS REFLEX

A low circulating volume (low blood pressure) is detected by the baroreceptors or pressoreceptors located mainly in the internal carotid artery (carotid sinus) and ascending aorta. They are also found in small quantities in the wall of almost every large artery in the neck and thorax[1]. These are pressure sensitive receptors which are physiologically inactivated when the aforementioned arteries become less stretched as a result of low blood pressure (Figure 1)[52]. Consequently, the carotid and aortic baroreceptors signal conduction to the cardio and vasomotor regulatory centers in the medulla oblongata, *via* the glossopharyngeal and vagus nerves, respectively, is subdued. Thus, the cardio-

vasomotor regulatory centers become more active and sequentially induce the sympathetic nervous system to become active while suppressing parasympathetic (vagus nerve) stimulation to the heart. The sympathetic nervous system through the cardiac accelerator nerve increases the heart rate and cardiac output. Furthermore, the adrenal medulla, under the influence of the sympathetic nervous system, releases both adrenaline and noradrenaline. This eventually leads to an increase in mean arterial pressure through increased cardiac output and peripheral vascular resistance by acting on adrenergic receptors. The sympathetic response is further augmented by the activation of the RAAS and the release of vasopressin[53]. Unfortunately, those complex neurohormonal compensatory responses to low blood pressure are temporary and the whole system that comprises the baroreceptors, RAAS and vasopressin becomes adapted and mal-responsive to the low circulating volume stimulus within 48-72 h[4]. Consequently all the above outlined responses are reversed resulting in profound hypotension, renal hypoperfusion and worsening renal failure.

RENIN ANGIOTENSIN ALDOSTERONE SYSTEM

Arterial hypotension and reduced blood flow to the kidney results in decreased sodium delivery to the macula densa which in turn causes the release of renin from the juxtaglomerular apparatus[54]. The renin release is the rate determining step for the activation of the RAAS. The activation of RAAS causes sodium and water reabsorption and vasoconstriction of the renal arteries (Figure 1)[55]. These compensatory mechanisms maintain effective circulation in the early stages of the disease (compensated) but with time their effects eventually become deleterious and lead to the development of complications. These include ascites, hyperkinetic circulation, nitric oxide release, renal failure, and increased venous capacitance with decreased venous compliance. Essentially all these culminate to form the bases of HRS[55].

Liver cirrhosis has been shown to be associated with activation of RAAS but also an increase in vasopressin secretion[56]. The activation of RAAS may contribute to decreased renal perfusion[56]. Importantly, albumin administration has been shown to be associated with a decrease in plasma renin level[57]. In contrast, administration of vasopressin does not substantially change renal perfusion, though it induces splanchnic vasoconstriction[57]. This may explain the potential benefit of administration of vasopressin and its analogues in HRS. The progression of PHT is associated with an increase in vasodilation of the splanchnic circulation and marked resistant to vasopressin. It is likely that further research is needed to address the interaction between RAAS and vasopressin.

In addition, the activation of the RAAS has an important role in hemodynamic regulation in the liver as well as proliferation of vascular smooth muscle cells and fibrosis through 2 well coordinated complementary pathways: (1) vasoconstriction/proliferative pathway, incorporating the angiotensin converting enzyme (ACE), angiotensin (Ang) Ⅱ-Ang Ⅱ type 1 (AT1) receptor; and (2) a counter- regulatory vasodilatation/antiproliferative pathway, involving ACE2-Ang-(1-7)-Mas receptor[58]. It is important to point out that the ACE, vasoconstriction/proliferation pathway induces contraction and proliferation of hepatic stellate cells, which lead to fibrosis[58]. In contrast, activation of ACE2-Ang-(1-7) is not only associated with liver cirrhosis-induced splanchnic and systemic vasodilatation but also anti-fibrotic effects[58].

Vilas-Boas *et al*[59] suggested that the administration of a combination of propranolol and ACE inhibitor or AT1 receptor blocker may have potential benefit in cirrhotic patients. Furthermore, administration of propranolol *per se* has been shown to be associated with unfavorable consequences on the 2 main RAAS components, Ang Ⅱ and Ang(1-7), in the splanchnic and peripheral circulation[59]. In contrast, inhibition of RAAS by ACE inhibitors and AT1 receptor blockers has been shown to be associated with potential benefit in slowing progression of liver fibrosis and even cardiac and renal fibrosis[60].

Interestingly, it has been hypothesized that the effect of Ang Ⅱ dominates in advanced liver disease while the effect of Ang(1-7) dominates in moderate liver disease[61]. Therefore, the RAAS can be viewed as a dual system that leads to vasoconstriction, fibrosis, vasodilatation and antifibrosis. However, further research is urgently needed to establish a therapeutic benefit of the dual function of the RAAS in targeting liver disease and preventing fibrosis in humans and, in particular, in the management of HRS.

DETRIMENTAL EFFECTS OF THE SYMPATHETIC NERVOUS SYSTEM AND RAAS

These wonderful compensatory mechanisms with prolonged activation result in even more increased sodium and water retention with a subsequent increase in total circulatory volume. RAAS, with its overall effect of salt and fluid retention contributes to the development of ascites usually in the presence of PHT. In addition, splanchnic vasodilation appears to be one of main culprits in the formation of ascites[50]. Ascites develops when there is increased sinusoidal pressure which forces fluid to leak into the abdominal cavity[1]. Ascites further fuels the constant activation of the RAAS and the sympathetic nervous system which fail to maintain effective circulating volume.

The lack of response to neurohormonal mechanisms in the latter stages of cirrhosis may be due to several factors: (1) nitric oxide-mediated pooling of blood in splanchnic vascular bed; (2) hyporesponsiveness of splanchnic vasodilation to neurohormonal mechanism; (3) ascites; and (4) downregulation of receptors.

Eventually the pooled blood in the splanchnic circulation cannot be utilized fully in the presence of ineffective

circulation. This is on account of the fact that the reservoir of blood in the splanchnic circulation continues to increase and is mediated by locally-produced nitric oxide. A recent study by Li *et al*[62] demonstrated that nitric oxide caused changes in mesenteric venous capacitance and increased pooling of blood in rats with liver cirrhosis. In this study it was shown that the cirrhotic rats had a nitric oxide-mediated increase in venous capacitance and decrease in compliance. Nitric oxide is produced locally by eNOS which is activated by the high shear stress in the splanchnic vascular endothelium, which is caused by the increased splanchnic blood flow. In both animal and human studies, nitric oxide appears to be the main orchestrator of the splanchnic vasodilation that facilitates the pooling of blood[63,64], *via* its direct action on the vascular smooth muscles.

Additionally, it is widely accepted that nitric oxide antagonizes the sympathetic and RAAS-driven vasoconstriction thus inducing vasodilatation of the splanchnic vascular bed. Accordingly, ineffective circulation continues to trigger neurohormonal responses, though in the latter stages of the disease there is hyporesponsiveness to these compensatory mechanisms. In the early stages of compensated liver cirrhosis, the neurohormonal activation is able to overcome the splanchnic vasodilation and maintain an acceptable circulating volume. However with decompensated liver cirrhosis; and increased PHT, the hyporesponsiveness is often an indicator of progression towards the end stage.

It is suggested that the hyporesponsiveness may be due to desensitization and downregulation of adrenergic receptors. In cirrhotic rats there appears to be β-adrenergic receptor hyporesponsiveness to catecholamines[65]. This concept is not new; previous studies looking at heart failure have shown that prolonged activation of the neurohormonal response leads to a downregulation of β-adrenergic receptors[66]. The hyporesponsiveness of the myocardium to catecholamine stimulation that is seen in cirrhosis is termed cirrhotic cardiomyopathy[67].

FUNCTIONAL RENAL FAILURE

The renal system, through autoregulation, maintains a physiologically acceptable GFR over a range of blood pressures. Autoregulation consists of myogenic and neurohormonal responses. The development of a hyperkinetic circulation results in renal hypoperfusion despite increased total circulating volume. Initially with low blood pressure, the kidneys respond with smooth muscle contraction in the vessels (myogenic response), which helps to maintain the perfusion pressure[1]. This response alone is not adequate, therefore the sympathetic nervous system and RAAS are activated and subsequently lead to renal vasoconstriction. The peripheral vasodilatation that occurs is perceived by the kidneys as a hypovolemia, that continues to promote renal vasoconstriction. The resultant effect is reduced GFR, oligo-anuria, raised plasma creatinine and the development of hepatorenal failure[2].

Renal biopsies in HRS patients have shown remarkably normal renal histology architecture despite the dismal renal function. The findings have led to the term "reversible functional renal failure" being coined[22]. The primary problem causing renal failure is liver cirrhosis and this functional renal failure can be reversed with liver transplantation. Kidney transplants from palliated patients with HRS into those with intrinsic renal failure, have remarkably been show to have reversed to normal renal function[68,69]. This further supports the concept of reversibility of functional renal failure in patients with HRS. The definitive treatment for HRS is liver transplantation; however with increasing shortages of organ donation and with long waiting lists, patients are more often than not succumbing to the dismal prognosis of HRS. Nevertheless treatment with vasopressin analogues, in particular terlipressin, has been shown to reverse the renal failure and can be used as a bridge to definitive treatment (liver transplant).

TERLIPRESSIN AS POTENTIAL TREATMENT FOR HRS

Terlipressin, an analogue of vasopressin, is used as potential treatment of HRS. In this review we focus on clinical trials and their strengths and weaknesses. It is worth mentioning that in the majority of these studies, terlipressin was used in combination with albumin. Furthermore, other trials compared the effect of terlipressin with noradrenaline. We included clinical trials with evidence-based medicine. Hence, the subsequent discussion will focus on the impact of terlipressin *vs* placebo and terlipressin *vs* noradrenaline with and without albumin.

Terlipressin and clinical trials

Terlipressin (without albumin) *vs* placebo: Hadengue *et al*[70] carried out a double-blind, crossover, randomized study in 9 patients with type 1 HRS. The patients received terlipressin (2 mg/d for 2 d) and a placebo for 2 d in a randomized order. Terlipressin administration significantly increased creatinine clearance and urine output, but did not significantly change urinary sodium concentration. Urinary sodium excretion was not significantly different after placebo administration or terlipressin administration. Terlipressin administration significantly decreased plasma concentrations of renin and aldosterone but not atrial natriuretic peptide levels, and these biochemical changes were not seen in the placebo group. The study by Solanki *et al*[19] was a randomized, controlled, single-blind trial. They assigned 24 consecutive patients with HRS to treatment with terlipressin 1 mg iv at 12 h intervals (group A, $n = 12$) or placebo at 12 h intervals (group B, $n = 12$). The end-point of the study was improvement in renal function defined as reversal of HRS and survival at 15 d. Terlipressin administration was shown to be associated with an improvement in parameters of renal function, mean arterial blood pressure and importantly reversal of HRS in 5 of the 12 patients in group A.

Table 1 Summary of effect of terlipressin associated with albumin on hepatorenal syndrome

Study	Main outcome
Sanyal *et al*[72], 2008	Terlipressin administration with albumin shown to be associated with improvement in renal function and appeared superior to placebo in reversing type 1 HRS
Martín-Llahí *et al*[73], 2008	Terlipressin administration with albumin shown to be associated with improvement in renal function in patient with liver cirrhosis and type 1 HRS, without significant impact on 3-mo survival
Neri *et al*[74], 2008	Terlipressin administration with albumin shown to be associated with improvement in renal function in patients with type 1 HRS and also a high probability of survival
Uriz *et al*[17], 2000	Terlipressin associated with albumin appeared to be a safe and effective treatment of HRS and decreased the frequent ischemic complications associated with terlipressin treatment alone. Terlipressin associated with albumin therapy was associated with marked improvement in renal function, reversal of HRS and improvement in circulatory function with an increase in mean arterial blood pressure

HRS: Hepatorenal syndrome.

Interestingly, Testro *et al*[71] reviewed outcomes of 69 patients treated with terlipressin between 2001 and 2005. Their findings showed that 49 episodes (71%) of HRS were type 1, and 20 episodes (29%) were type 2. Forty-one (59.4%) patients responded to terlipressin. Twenty-one (30.4%) patients survived; 17 (81%) had type 1 HRS while 4 (19%) had type 2 HRS ($P = 0.27$). The only factor predicting transplant-free survival was type 1 HRS. No patients with type 2 HRS survived without transplantation ($P = 0.02$). These trials clearly showed the potential benefit of administration of terlipressin in individuals with HRS. However, Terlipressin administration was associated with minimal reversible ischemic events e.g. crampy abdominal pain and cardiac arrhythmias. We suggest that randomized clinical trials are now warranted.

Terlipressin (with albumin) *vs* placebo: Interestingly, concomitant administration of terlipressin and albumin is shown to be associated with better clinical outcomes. A summary of studies that used terlipressin (with albumin) *vs* placebo is provided in Table 1.

Terlipressin *vs* noradrenaline: The use of noradrenaline, a cheap and widely available drug, in the management of HRS was shown to be as effective as terlipressin but associated with increased risk of ischemic events. Data from an unblinded, pilot study suggested that noradrenaline was as effective and safe as terlipressin in patients with HRS. Twenty-two consecutive cirrhotic patients with HRS (9 with type 1 HRS; 13 with type 2 HRS) were randomly assigned to treatment with noradrenaline (0.1-0.7 μg/kg per minute) and albumin (10 patients) or with terlipressin (1-2 mg/4 h) and albumin (12 patients). Treatment was administered until HRS reversal or for a maximum of 2 wk. Reversal of HRS was observed in 7 of the 10 patients (70%) treated with noradrenaline and in 10 of the 12 patients (83%) treated with terlipressin. Treatment led, in both groups, to a significant improvement in renal and circulatory function; no patient developed signs of myocardial ischemia[75]. Sharma *et al*[76] reported similar beneficial results in treating HRS with noradrenaline, however, they also reported that 2 patients had ventricular ectopies with noradrenaline. We suggest that further studies are urgently needed to evaluate the use of noradrenaline as potential treatment for HRS.

Terlipressin and meta-analyses

Several meta-analyses have been conducted to determine the effect of terlipressin in HRS with regard to the duration of treatment, infusion of albumin and comparison with noradrenaline. Dobre *et al*[77] concluded in their meta-analysis that terlipressin administration was associated with improvement in HRS reversal and that noradrenaline has the same effect as terlipressin in improving surrogate markers of HRS. Sagi *et al*[11] showed that the risk ratio for reversal in type 1 HRS with terlipressin therapy was 3.66 [95% confidence interval (CI): 2.15-6.23]. Recurrence of HRS was low (8%). Serious side effects requiring discontinuation of therapy were seen only in 6.8% of patients on terlipressin therapy. There was a trend towards improved transplant-free survival at 90 d in the Terlipressin group (relative risk 1.86, 95% CI: 1.0-3.4, $P = 0.05$). The conclusion of their meta-analysis was that terlipressin is effective in reversing HRS type 1 and recurrence of HRS is rare with at least 14 d of therapy and associated with an increased survival. Importantly, Fabrizi *et al*[78] showed in their meta-analysis that discontinuation of terlipressin therapy was associated with a significant increase in the number of relapses. Furthermore, Fabrizi *et al*[79], in another meta-analysis, showed that terlipressin was more effective in reversing HRS than placebo without apparent impact of terlipressin on survival in HRS patients. This may again suggest the need for large clinical trials addressing the impact of terlipressin in HRS patient survival. Interestingly, administration of albumin with terlipressin showed a reduction in mortality in type 1 HRS[10]. Therefore, the current evidence suggests that terlipressin can have a potential benefit in treating HRS and that an improvement in survival can be achieved with its concomitant administration with albumin.

CONCLUSION

HRS continues to be a challenging task to manage following chronic liver cirrhosis. The grave prognosis and the short survival times have fuelled great interest in clinical

trials; its reversibility creates scope for prolonging both survival and quality of life. The current literature reviewed has further re-enforced terlipressin as a potential first-line treatment in HRS**.** Terlipressin has so far shown to increase survival rates and reverse functional renal failure. The increased neurohormonal response, especially of the RAAS, has been decreased with the administration of terlipressin. This subsequently improves circulatory dysfunction and lowers plasma creatinine levels near to baseline values. The effects of nitric oxide, which is a factor in the deleterious neurohormonal response, appears to be overcome by the administration of terlipressin through unknown mechanisms.

In addition, terlipressin has few adverse side effects, which allows patients to continue with treatment in order to achieve desirable effects. At present however, we acknowledge that there are a limited number of randomized, controlled studies carried out on terlipressin and therefore there is a real need for large multi-centered trials to be carried out. We recommend that terlipressin, with concomitant administration of albumin, may be the first line treatment in the management of HRS.

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EDITORIAL

Growth factor- and cytokine-driven pathways governing liver stemness and differentiation

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Abstract

Liver is unique in its capacity to regenerate in response to injury or tissue loss. Hepatocytes and other liver cells are able to proliferate and repopulate the liver. However, when this response is impaired, the contribution of hepatic progenitors becomes very relevant. Here, we present an update of recent studies on growth factors and cytokine-driven intracellular pathways that govern liver stem/progenitor cell expansion and differentiation, and the relevance of these signals in liver development, regeneration and carcinogenesis. Tyrosine kinase receptor signaling, in particular, c-Met, epidermal growth factor receptors or fibroblast growth factor receptors, contribute to proliferation, survival and differentiation of liver stem/progenitor cells. Different evidence suggests a dual role for the transforming growth factor (TGF)-β signaling pathway in liver stemness and differentiation. On the one hand, TGF-β mediates progression of differentiation from a progenitor stage, but on the other hand, it contributes to the expansion of liver stem cells. Hedgehog family ligands are necessary to promote hepatoblast proliferation but need to be shut off to permit subsequent hepatoblast differentiation. In the same line, the Wnt family and β-catenin/T-cell factor pathway is clearly involved in the maintenance of liver stemness phenotype, and its repression is necessary for liver differentiation during development. Collectively, data indicate that liver stem/progenitor cells follow their own rules and regulations. The same signals that are essential for their activation, expansion and differentiation are good candidates to contribute, under adequate conditions, to the paradigm of transformation from a pro-regenerative to a pro-tumorigenic role. From a clinical perspective, this is a fundamental issue for liver stem/progenitor cell-based therapies.

Key words: Hepatocyte growth factor; Epidermal growth factor; Fibroblast growth factor; Transforming growth factor-β; Hedgehog and β-catenin; Liver; Stem cell

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INTRODUCTION

Liver is unique in its capacity to regenerate in response

to injury or tissue loss. Following two-thirds partial hepatectomy, hepatocytes exit the G0 phase of the cell cycle and synchronously re-enter the cell cycle to regenerate the liver mass completely in 6-7 d in rodents, or 3-4 mo in humans[1-4]. Liver stem/progenitor cells do not seem to be required for this process. However, when this response is impaired, as in the case of a hepatocyte-selective proliferative defect or acute liver failure, the contribution of liver progenitors becomes much more relevant[5,6]. A population of small cells with a low cytoplasmic/nuclear ratio that arose from a small number of portal cells, not from hepatocytes, were first observed in rodents and became known as "oval cells"[7,8]. Although several lines of evidence suggest that oval cells derive from the biliary compartment, other origins have been also suggested[4].

Significant work in the past few years has focused on the signaling pathways, as well as cell-cell interactions, that control the initial proliferation/expansion and the terminal differentiation of liver stem/progenitor cells. Oval cells express MET, the receptor of the hepatocyte growth factor (HGF). The superabundance of HGF-producing cells in the immediate vicinity of oval cell proliferation and differentiation suggests that this growth factor is involved in all aspects of stem cell behavior, proliferation, migration, and differentiation, through a paracrine mechanism[9]. Oval cells also express receptors for epidermal growth factor (EGF)-like ligands, and *in vivo* infusion of a combination of HGF and EGF enhance the mitogenic response of oval cells after administration of 2-acetylaminofluorene[10], which reveals the relevance of both growth factors in liver stem/progenitor cell biology. Additionally, different studies have revealed that oval cells also respond to other growth factors in an autocrine/paracrine manner[11]. The transforming growth factor (TGF)-β family of cytokines play a relevant role in the maintenance of embryonic stem cell identity, and it has been shown that the specification of pancreas and liver progenitors is restricted by the TGF-β pathway[12]. All these growth factors and cytokines might modulate not only proliferation of liver stem/progenitor cells, but also cell death, as well as contributing to their terminal differentiation.

This review gives an update on recent relevant studies of the growth factors and cytokine-driven intracellular pathways that govern liver stem/progenitor cell expansion and differentiation, and the relevance of these signals in liver development, regeneration and carcinogenesis.

TYROSINE KINASE RECEPTOR-MEDIATED SIGNALING PATHWAYS

HGF

HGF was firstly identified in the 1980s as a potent mitogen for hepatocytes[13-15]. A factor secreted by fibroblasts and smooth muscle cells was discover separately, which promoted epithelial cell scattering[16]. Later studies unraveled that HGF and scatter factor were indistinguishable[17,18]. HGF is a growth factor that induces a wide range of biological activities, including stimulation of proliferation, migration, morphogenesis, and survival of a variety of cell types[19-24], which plays a major role in tissue formation and homeostasis. HGF acts through binding to its tyrosine kinase receptor, Met. Ligand-receptor binding results in autophosphorylation of the receptor in specific tyrosine residues located in the C-terminal domain, and subsequent phosphorylation/activation of multiple adapter and signal transducing proteins, such as growth factor receptor-bound protein 2 (Grb2)/Sos, Ras-mitogen-activated protein kinase, Grb2-associated binding protein 1 (Gab1), phosphoinositide 3-kinase (PI3K), phospholipase C-γ, p38, and signal transducer and activator of transcription (STAT)-3, among others, which mediate the biological activities of HGF/c-Met[25,26]. For decades, HGF has been recognized as a growth factor involved in the hepatocyte proliferative response during liver regeneration (a recent review on the role of HGF in liver regeneration can be found in[3]), but an unequivocal demonstration of an essential role of the HGF/c-Met signaling in liver regeneration has only been provided recently. Thus, liver specific c-Met and HGF conditional knock-out mice show an impairment of the regenerative response[27-29]. Hepatocytes that lack a functional c-Met display reduced basal survival and a higher sensitivity to Fas-induced liver damage, both *in vivo* and *in vitro*. Moreover, after toxic liver injury induced by exposure to CCl_4, c-met$^{-/-}$ livers showed delayed healing from necrotic injury[28]. Complete abolition of the cell cycle has also been demonstrated when c-Met is deleted after partial hepatectomy, by using RNA interference techniques[30]. In addition to the effects on proliferation, these authors have described an alteration in expression of apoptosis-related genes, particularly, increased expression of pro-apoptotic genes, and decreased expression of anti-apoptotic genes, and enhanced activation of caspase 3. Altogether, these studies provide clear evidences of a role for this ligand/receptor system in promoting hepatocyte proliferation, survival, and tissue remodeling during liver regeneration.

HGF/c-Met signaling is also essential during fetal liver development. Both HGF- and c-Met-deficient embryos show abnormally small livers and liver-to-body weight ratios and massive hepatocyte apoptosis[31-33]. Recent studies from Dr. Maina's laboratory have demonstrated HGF/c-Met survival properties in primary embryonic hepatocytes, as shown by the ability of HGF to impair Fas-induced apoptosis by acting through PI3K and AKT to prevent FLICE inhibitory protein degradation[34]. Additionally, in the context of the adult liver, numerous works have reported an important contribution of the pro-survival activity of HGF in protecting liver during fibrosis and other hepatic dysfunction[35-40], thus expanding the scenarios in which the anti-apoptotic activity of HGF plays an active role.

Although hepatocytes have long been considered the prime target of the actions of HGF, there is accumulating evidence of an important role for HGF/c-Met signaling on liver stem/progenitor cell function and behavior. Liver stem/progenitor cells express c-met[41]. Furthermore,

during oval cell activation induced by N-acetyl-2-aminofluorene/partial hepatectomy (AAF/PH) in rats, HGF expression increases coincidentally with oval cell proliferation, mainly on the periportal regions where oval cells are located[9,41]. These data suggest that the HGF/c-Met system regulates some aspects of liver stem/progenitor cell biology. In support of this, *in vivo* infusion of HGF during AAF/PH-induced liver regeneration stimulates oval cell expansion into the liver lobules[10]. Similar results have been obtained by *in vivo* transfer of HGF cDNA into liver subjected to the Solt-Farber regime[42]. HGF-dependent mitogenic activity has also been shown in rat and mouse oval cell lines *in vitro* by either adenovirus-mediated transfer of the HGF gene or addition of exogenous HGF[43-46]. The molecular mechanisms that mediate the mitogenic effects of HGF in liver progenitors appear to be cell-type specific, because PI3K/AKT activation[43] and nuclear factor-κB activation, downstream of p38 and extracellular signal-regulated kinase (ERK) MAPKs[44], are involved. Additionally, bi-potential hepatoblast cell lines (precursors of both hepatocytes and cholangiocytes) have been established from transgenic animals expressing a constitutively active human Met[47]. However, HGF is much more than a mitogen for liver stem/progenitor cells. HGF effectively protects WB-F344 cell from apoptosis induced by tumor necrosis factor-α in a dose-dependent manner[44]. More recently, using a novel *in vitro* model of genetically modified oval cells that harbor an inactivated Met tyrosine kinase, we have demonstrated that loss of Met increases sensitivity to apoptosis caused by serum deprivation or treatment with TGF-β[46]. By virtue of these results, we hypothesize that Met-driven anti-apoptotic activity plays an important role supporting the expansion of liver progenitors following liver injury, by helping them to overcome the local tissue injuries and inhibitory signals. Therefore, the HGF/c-Met signaling pathway might be a major survival pathway in liver that operates during liver development, homeostasis, and regeneration (both hepatocyte and oval cell-mediated).

The role of HGF as a morphogen is already established[48]. Morphogenesis is a complex invasive growth program that plays a fundamental role in normal development[49,50]. Not surprisingly, morphogenic properties of HGF are exhibited in embryonic and postnatal mouse non-parenchymal epithelial cell-derived cell lines. In these cells, HGF induces a morphogenic response that includes cell scattering and ductal branching in collagen gels. These changes are not observed by treatment with other liver growth factors including EGF, TGF-β, acidic fibroblast growth factor (aFGF) and insulin, and are not inhibited by TGF-β[51]. A similar response has also been described in pancreatic oval cells *in vitro*; a cell population closely related to its hepatic counterpart[52]. Recently, HGF has been directly involved in promoting motility and invasiveness of human liver progenitor cells through Matrigel; an effect that is partially mediated by matrix-metalloproteinase-mediated extracellular matrix (ECM) proteolytic degradation and activation of the MAPK/ERK pathway[53].

Another important biological activity of HGF in liver progenitor cells that cannot be overlooked is its modulatory effect on the differentiation capacity of these cells. HGF has been reported to be required not only for an efficient proliferation and survival, but for hepatocytic differentiation of embryonic hepatic stem cells *in vitro*[54-57]. Furthermore, virtually all the strategies for *in vitro* differentiation of embryonic and adult stem/progenitor cells of different origin into hepatocytes include HGF as a hepatic-inducing factor (a thorough compilation of *in vitro* differentiation methods is found in[58]). Successful differentiation is generally achieved by step by step addition of growth factors, cytokines, and hormones, trying to emulate the sequence of events taking place during *in vivo* hepatogenesis. The requirement of HGF in this process relies on the specific role played by this growth factor at different liver developmental stages. During the commitment phase, HGF might antagonize differentiation of bipotential hepatoblasts along the cholangiocytic lineage, which results in support of growth and differentiation of fetal hepatocytes[59]. In fact, results from Suzuki *et al*[57] have demonstrated that HGF can initiate differentiation of albumin-negative liver stem cells into albumin-positive hepatic precursors at the same time that allows their expansion and decreases expression of cholangiocyte-lineage markers, such as CK19 or γ-glutamyl transferase, but it cannot induce latter markers of hepatocyte differentiation, such as glucose-6-phosphatase or tryptophan-2,3-dioxygenase.

Later, after birth, the expression of both HGF and c-Met significantly increases in the liver, and activation of this pathway crucially assists during complete functional hepatic maturation[41,60]. We have also described that HGF combined with TGF-β helps to maintain the expression of hepatocyte differentiation markers in rat fetal hepatocytes in culture[61,62]. In rat primary neonatal hepatocytes, HGF promotes scattering, but this effect is not associated with a dedifferentiation process, as shown by an increase in the expression of hepatic differentiation markers using HGF treatment[63,64]. Although a role for HGF in hepatocyte differentiation seems to be beyond reasonable doubt, it should be pointed out that HGF has also been directly implicated in transdifferentiation of hepatocytes to biliary epithelial cells[65]. These data highlight the enormous complexity in terms of signaling networks and molecular mechanisms associated with regulation of phenotypic transitions in liver epithelial cells.

All these results provide sufficient evidence to support a crucial role for an HGF/c-Met-induced signaling pathway in liver stem/progenitor cell biology. What it is not totally clear, however, is its mode of action. HGF is mainly produced by mesenchymal cells, and c-Met is expressed in epithelial cells, therefore, this ligand-receptor system is generally considered to act in a paracrine fashion[66]. Consistent with this, the main producers of HGF in the liver are the stellate cells[67,68], although sinusoidal endothelial cells also express HGF[69]. c-Met is expressed in hepatocytes, biliary epithelial cells, and liver progenitor cells[41,54,70,71]. As men-

tioned above, in rat models of oval cell activation *in vivo*, HGF mRNA has been identified in the desmin-positive stellate cells that surround the ductal structures of oval cells, or interspersed with the oval cells, whereas c-Met expression is detected in oval cells[9,41]. These results have prompted the conclusion that the HGF/c-Met system operates in oval cells *via* a paracrine mechanism. However, *in vitro* studies carried out in our laboratory in oval cell lines have shown an autocrine regulatory mechanism for HGF/c-Met involved in protection against apoptosis[46]. Whether the autocrine regulation plays a role *in vivo* has not been explored, but these data suggest that oval cells might respond to both autocrine and paracrine Met signaling in a context-dependent fashion.

EGF receptor ligands

Another tyrosine kinase receptor family that regulates liver pathophysiology is the EGF receptor (EGFR) family. This receptor is part of a complex signaling system that includes multiple ligands, namely TGF-α, EGF, heparin-binding EGF, amphiregulin, betacellulin, epiregulin, epigen, and crypto; and four transmembrane receptors: EGFR (Her1/ErbB-1), ErbB-2 (Her2/neu), ErbB-3 (Her3), and ErbB-4 (Her4). Complexity of this pathway relies on differential ligand binding affinity to the receptors, as well as formation of receptor homo- and heterodimers, all of which leads to activation of distinct intracellular signaling cascade and diverse biological activities. Among all the ligands, TGF-α and EGF are the most widely studied. Both TGF-α and EGF bind and activate the same receptor, EGFR. Ligand binding results in dimerization and autophosphorylation of EGFR in tyrosine residues of the cytoplasmic domain. This leads to recruitment of adapter proteins, such as Grb2 and Shc, and subsequent activation of multiple downstream pathways, including PI3K, Ras-MAPK, c-Jun N-terminal kinase, p38, protein kinase C and STAT-3, which mediate cell proliferation, migration, differentiation and evasion from apoptosis[72-76]. TGF-α and EGF are well-known regulators of hepatocyte proliferation. Infusion of either one of the two factors initiates DNA synthesis in liver of adult rats[77,78], and when added exogenously, they stimulate growth of primary hepatocytes at all developmental stages: fetal, neonatal, and adult[79-82]. Together with HGF, EGFR ligands represent the only complete mitogens in adult hepatocytes in serum-free medium, and the most important growth factors involved in the proliferative response during liver regeneration[3]. Demonstration of a critical role for EGFR in hepatocyte proliferation during the initial phases of liver regeneration has recently been provided by generating mice with a liver-specific EGFR deficiency[83]. In addition to the regulatory role in proliferation, and similarly to Met, EGFR is a major survival pathway in the liver[84]. EGF-mediated EGFR activation is able to abolish completely the apoptotic response induced by TGF-β in fetal rat hepatocytes[62] or by Fas receptor stimulation in mouse hepatocytes[85]. Furthermore, EGFR ligands appear to be important modulators of hepatocyte differentiation as well. Morphological and gene expression studies from our laboratory and collaborators have shown that EGF, acting in cooperation with other cytokines and hormones, maintains primary fetal and neonatal hepatocyte differentiation[64,86,87].

Expression studies have suggested that ErbB1-triggered signaling also plays a role in stem/progenitor-cell-mediated liver regeneration. Indeed, both TGF-α and EGFR are transcriptionally upregulated during the period of active proliferation and differentiation of progenitor cells in the rat liver subjected to the 2-AAF/PH protocol[88], and they appear to drive the early proliferation of the progenitor cell compartment[89,10]. Although both HGF and EGF promote the expansion of oval cells *in vivo*, some differences are observed. HGF increased number of both ductal and Ito cells at a similar rate, whereas infusion of EGF mostly increases ductal cells. Our laboratory and others have also shown that EGF and TGF-α are mitogens in mouse and rat oval cells *in vitro*[11,46,90].

The effects of this signaling pathway on liver stem/progenitor cells are not restricted to mitogenesis. *In vivo* infusion with either EGF or HGF not only amplifies liver progenitor expansion following liver injury but also decreases apoptosis[10]. We also have evidence of an important role for EGFR-mediated signaling in regulating oval cell survival *in vitro*. Thus, inhibition of EGFR *via* treatment with a synthetic inhibitor increases basal apoptosis (in the absence of serum and exogenous stimuli) and strongly amplifies TGF-β-induced apoptosis (our unpublished results). EGFR-mediated motogenic, morphogenic, and differentiation activities have also been reported. EGF, combined with TGF-β, triggers a scattering response in mouse oval cell lines *in vitro*[11]. In addition to this, EGF is often included as a hepatogenic factor in strategies to induce *in vitro* differentiation of stem/progenitor cells into hepatocyte-like cells[58]. Consistently, TβT-FH cell lines - a cell population of fetal hepatocytes that has suffered an epithelial mesenchymal transition (EMT) and a dedifferentiation process after TGF-β treatment - recover the original epithelial phenotype and gain cytokeratin-19 expression by treatment with EGF and DMSO, which suggests conversion to hepatoblast-like cells[91].

It should be noted that we have seen autocrine regulation for EGFR-dependent signaling in oval cell lines (our unpublished results). Different to the HGF/c-Met system, an EGFR-ligand-mediated autocrine mechanism has been suggested, based on the detection of EGFR ligand transcripts in oval cells during liver regeneration[88]. *In vivo* and *in vitro* studies have shown that choline-deficient ethionine-supplemented diet-treated mouse livers express cytokines such as lymphotoxin-β, interferon-γ, and interleukin (IL)-6[92,93]. Collectively, these data strongly support that autocrine regulatory mechanisms are important in liver progenitor cells. The significance of the c-Met and EGFR autocrine loops in oval cells is not yet totally understood. Certainly, autocrine signaling has been mostly associated with malignancy. This seems to apply to stem/progenitor

cells as well, because tumorigenic conversion of mouse oval cell has been associated with growth factor production and alteration in growth factor responsiveness[11]. More specifically, an HGF/c-Met autocrine loop has been identified in spontaneously transformed WB-F344 rat liver stem-like cells, which contributes to drive autonomous cell proliferation[94]. Spontaneously transformed oval cells express TGF-α. These cells form tumors when injected into nude mice, a capacity that is significantly reduced by transfection with TGF-α antisense gene[95]. A cross-talk between Met and EGFR has also been proposed. Thus, rat liver-epithelial-cell-derived tumor cell lines that constitutively express TGF-α display increased levels of both c-met gene and protein, as well as an amplified response to HGF[96]. However, 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-treated mouse liver-derived oval cell lines, which show autocrine activation of Met and EGFR-dependent pathways, do not show any sign of transformation[46] (and our unpublished results). Whether this is due to a matter of time, dose, or lack of critical partners for the neoplastic transformation, it is not yet known. For now, awaiting further conclusive experiments, a direct link between the establishment of functional c-Met and EGFR-dependent autocrine loops and neoplastic transformation cannot be firmly established in liver progenitor cells.

All the data summarized above highlight not only a relevant role of Met and EGFR-triggered signaling pathways in regulating the liver progenitor cell compartment, but a striking parallelism between the two pathways, both of which mediate growth, survival, migration and hepatocytic differentiation (Figure 1). Further studies will clarify which of these biological activities are totally overlapping and which are not. In this sense, Met and EGFR mutant mice and cell models should provide very useful and invaluable tools in this regard.

FGFs

The FGFs are a family of growth factors with high affinity for heparan sulfate proteoglycans, which bind to transmembrane tyrosine kinase FGF receptors (FGFRs). The role of these growth factors in hepatic fate specification of pre-hepatic endoderm cells during liver development is well known. There are a number of excellent reviews on this subject, some of which are cited here[97-99]. Activation of adult stem/progenitor cells is believed to use similar, if not identical, genetic programs as the embryonic progenitors, therefore, FGF is among the main targets to be analyzed in rodent models of oval cell expansion. Indeed, aFGF (also known as FGF-1) is upregulated at the stage of oval cell progression[89,100], being expressed by both oval cells and Ito cells. FGFR1 and FGFR2 are also expressed at high levels during the period of active proliferation and differentiation of oval cells, but exhibit a different pattern. Although FGFR1 is mainly expressed in oval cells, FGFR2 is expressed in both oval and Ito cells[101]. These results suggest a differential role for these two receptors during liver stem/progenitor-cell-mediated regeneration, as well as the establishment of both autocrine and paracrine signaling. In addition to these *in vivo* observations, *in vitro* studies have shown that aFGF is able to push Met murine hepatocytes-bi-potential precursors isolated from transgenic livers, which express a constitutively active human Met - to progress from a very early state of differentiation to a more mature state, associated with the expression of liver functions[47]. Moreover, FGF1-pretreated cells are resistant to the TGF-β dedifferentiation effect[102]. Essentially, FGFs have proved to be effective in mediating early hepatic differentiation, and therefore are included in most *in vitro* differentiation protocols[58].

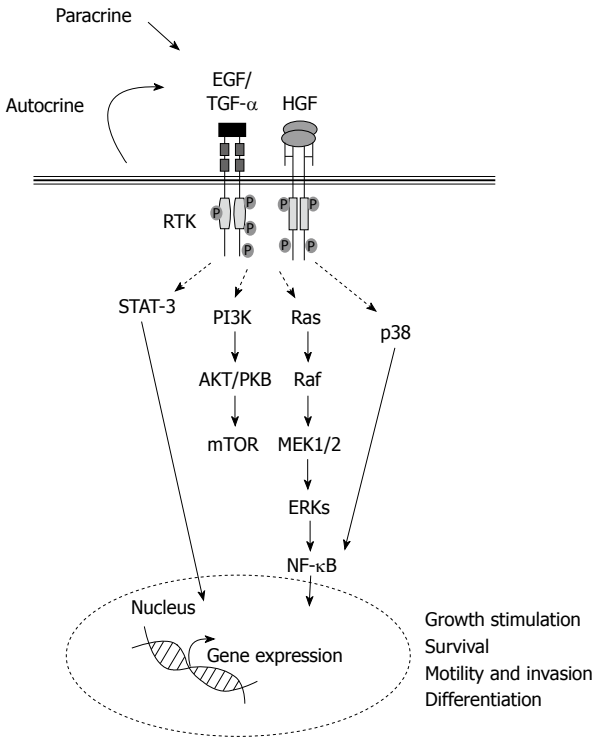

Figure 1 Schematic illustration of the major signaling pathways and biological activities induced by hepatocyte growth factor and epidermal growth factor receptor ligands in liver progenitor cells. HGF: Hepatocyte growth factor; EGF: Epidermal growth factor; TGF: Transforming growth factor; STAT: Signal transducer and activator of transcription; PI3K: Phosphoinositide 3-kinase; NF-κB: Nuclear factor κB; mTOR: Mammalian target of rapamycin; MEK: Mitogen-activated protein kinase kinase; ERKs: Extracellular-signal-regulated kinases.

TGF-β FAMILY

The TGF-β family of cytokines regulates hepatocyte proliferation and death, and plays relevant roles during liver regeneration[103]. However, TGF-β and other pro-inflammatory cytokines are important inducers of fibrocarcinogenesis, due to their ability to induce myofibroblast differentiation and ECM deposition[104,105]. Recent evidence has shown that TGF-β might also regulate liver stemness and phenotype[106].

TGF-β active form acts as a dimer, and signals by bringing together two receptors with serine-threonine kinase activity, which are known as type I and type II receptors (Figure 2). After TGF-β binding, the type II receptor

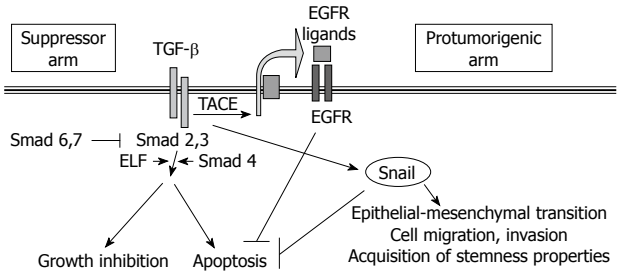

Figure 2 Overview of the major signaling pathways, and their cellular effects, induced by transforming growth factor-β in liver cells. TGF: Transforming growth factor; EGFR: Epidermal growth factor receptor; ELF: Embryonic liver fodrin; TACE: Tumor necrosis factor-converting enzyme.

phosphorylates and activates the type I receptor, which is responsible for phosphorylation of the receptor-regulated Smad family of transcription factors (R-Smads), i.e. Smad 2 and 3 in the case of TGF-β1, β2 and β3[107]. After phosphorylation, Smad 2 or 3 forms a complex with Smad 4 and shuttles to the nucleus, where it possesses DNA-binding activity, although it must associate with other DNA-binding co-factors to achieve high-affinity binding. Indeed, through combination with different transcription factors, the same TGF-β stimulus can induce or repress many different target genes. Activation of the Smad transcriptional activity can be counteracted by the presence of other members of the Smad family that play inhibitory effects (inhibitory Smads), such as Smad 6 and Smad 7, which compete with Smad 2 and 3 for binding either to the receptor complex or to Smad 4.

The most relevant Smad-mediated cell responses to TGF-β are growth inhibition and apoptosis. TGF-β inhibits proliferation in adult[108,109], as well as fetal or regenerating hepatocytes[82,109], and it is a well-known inducer of hepatocyte cell death[110,111]. The inhibitory effects of TGF-β on liver stem/progenitor cell growth and survival are somewhat unclear. TGF-β overexpression results in impairment of oval cell expansion induced by DDC[112]. Consistently, we have shown that treatment with TGF-β inhibits growth and induces apoptosis in DDC-derived oval cell lines *in vitro*[46]. However, when compared to adult hepatocytes, other authors have reported that the majority of the adult liver progenitor cells are more resistant to endogenously produced TGF-β anti-proliferative and pro-apoptotic effects *in vitro*[113], which indicates a differential sensitivity to TGF-β between liver progenitors and mature hepatocytes. Indeed, several mechanisms have been proposed that would explain this differential response. On the one hand, the ratio between R-Smads and inhibitory Smads might be different in oval cells. In this sense, Nguyen *et al*[114] have recently reported that oval cell lines show higher levels of Smad 6 than do adult hepatocytes. On the other hand, oval cells might show over-activation of survival signals, such as the MAPK/ERK pathway[115] that might be related to the autocrine production of growth/survival factors, such as HGF[46]. It is worth noting that, when combined with growth factors such as EGF or HGF, TGF-β might contribute to cellular scattering and morphological differentiation of hepatoblasts and liver stem cells[87,116,117]. In this same line of evidence, hepatocytes are undifferentiated and liver architecture is lost in Smad 2+/- and Smad 3+/- mice, as well as in ELF (a β-spectrin that is crucial for the propagation of the TGF-β signal) mutants[118-121]. Defects in Smad 2+/- and Smad 3+/- mutants are restored by HGF treatment. Although mechanisms by which TGF-β signals can be supplanted by HGF are unknown, data seem to support cooperation between two independent pathways, that converge on β1-integrin expression, rather than direct pathway crosstalk[119]. Emerging new data have reinforced the idea that TGF-β is important for stem cell transitioning to a progenitor cell phenotype, and ultimately, its conversion to a fully differentiated phenotype. Indeed, TGF-β signaling, and particularly ELF, appears to play a crucial role in hepatocyte proliferation and transitional phenotype during human liver regeneration, and its loss is associated with activation of liver progenitor cells[106]. It has also been shown recently that TGF-β might be a determinant in the appearance of progenitor/stem cells in hepatocarcinogenesis. The examination of human hepatocellular carcinoma (HCC) has revealed that cells that are labeled with stem-cell markers have unexpectedly lost the TGF-β receptor Ⅱ and ELF, and show marked activation of the IL-6 pathway, a major stem-cell signaling pathway[121,122]. These data support that absence of TGF-β-driven epithelial differentiation favors carcinogenesis.

However, TGF-β is a pleiotropic cytokine inducing several, and sometimes contradictory, signals in epithelial cells. Indeed, in addition to the well-known Smad-mediated transcriptional responses that predominantly address tumor suppressor actions, TGF-β induces other Smad-dependent or independent effects that contribute to tumor progression[107]. Among these, related to the topic of this review, one of the more relevant is the capacity to induce EMT processes. EMT is a physiological process during embryogenesis, in which an epithelial cell loses expression of adhesion molecules, such as E-cadherin, and other components responsible for cell polarity. Instead, they express mesenchymal components of the cytoskeleton and acquire motility and scattering properties[123]. A closely related phenotypic conversion is also detected in fibrosis and neoplasia and is associated with disease progression[124]. Members of the TGF-β family can initiate and maintain EMT in a variety of biological systems and pathophysiological situations, through activation of major signaling pathways and transcriptional regulators integrated in extensive signaling networks[125,126]. In culture, hepatocytes and hepatoma cells undergo EMT in response to TGF-β[127-130], which support a potentially crucial role for TGF-β in the development and progression of hepatic fibrogenesis and cancer. The mechanisms that allow cells to escape from the apoptotic effects of TGF-β and undergo EMT are not completely understood. However, recent results have indicated that, in some epithelial cells, including fetal rat hepatocytes and hepatoma cells,

TGF-β might induce both pro- and anti-apoptotic signals and their balance defines the cell fate[129,131-133]. In fact, certain evidence indicates a crosstalk between the genetic programs that control TGF-β-induced growth arrest/apoptosis and those that regulate EMT, because once the cell has adopted a mesenchymal phenotype, it does not respond to TGF-β suppressor effects[128,134,135]. Thus, TGF-β might regulate its own signaling to switch from tumor suppression to tumor progression. Indeed, it is interesting to point out that transcription factors of the Snail family, repressors of the E-cadherin gene, are required for cell survival during EMT[136,137].

In recent works from our group, we have demonstrated that transdifferentiation of liver cells from an epithelial to a mesenchymal phenotype, such as that mediated by TGF-β, induces dedifferentiation and allows enrichment in a population of cells with putative liver progenitor properties[91,128,138]. The phenotypic characteristics observed (elongated phenotype, strong expression of Thy-1, vimentin or α-smooth muscle actin) are also highly reminiscent of myofibroblasts[139]. These finding are in agreement with the recently proven fact that EMT not only endows cells with migratory and invasive properties, but also induces stem cell properties[123]. Furthermore, additional works have proved the capacity of TGF-β to induce/maintain a stemness phenotype in other tissues[140]. Preliminary results have indicated that human fetal hepatocytes respond to TGF-β downregulation of E-cadherin and upregulation of Snail, thus acquiring a fibroblastic-like morphology and a liver progenitor cell phenotype (unpublished observations from our group in collaboration with Dr. N. Fausto, University of Washington, Seattle, USA).

These findings might have implications for regenerative biology of the liver and open new perspectives for the *in vitro* isolation of putative liver stem/progenitor cells to be used in basic and translational research in the liver. Moreover, a recent study has also suggested that TGF-β treatment of HCC cells might induce the selection of cells expressing high levels of CD133, a putative stem-cell marker in diverse hematopoietic and non-hematopoietic tissues and cancers[141], through a mechanism partially dependent on the Smads pathway, and that involves epigenetic regulation of the CD133 promoter. These results indicate that TGF-β might also play a role in transdifferentiating liver tumor cells to liver cancer stem cells.

In the embryo, progenitors to pancreatic β cells and hepatocyte lineages arise from neighboring domains of ventral foregut endoderm. It has been recently found that the specification of pancreas and liver progenitors is restricted by the TGF-β pathway[12]. It has been speculated that TGF-β signaling restrains lateral endoderm specification until the cells move sufficiently far from the heart and into a bone morphogenetic protein signaling domain, with the latter then becoming the dominant Smad 4-dependent pathway, which leads to pancreatic induction. TGF-β signaling appears to be strongly inhibitory to the pancreatic lineage and modestly inhibitory to the liver lineage, which suggests that TGF-β also plays a relevant role in maintaining the stemness phenotype of endodermal precursors during liver development.

All these results suggest a dual role for the TGF-β signaling pathway in liver stemness and differentiation. On the one hand, TGF-β mediates progression of differentiation from a stem or progenitor stage, but on the other hand, it contributes to the expansion of liver stem cells. Further work is necessary to understand better the relevance of both effects in liver development, regeneration and carcinogenesis.

HEDGEHOG FAMILY LIGANDS

Hedgehog (Hh) family ligands are widely acknowledged morphogens that regulate tissue remodeling during embryogenesis[142,143]. In particular, Indian hedgehog and Sonic hedgehog ligands, and their receptor, Patched, are expressed at different stages of liver organogenesis, and available data point to dynamic signal activation and temporarily restricted effects on hepatoblast regulation. Thus, Hh signaling is necessary to promote hepatoblast proliferation but it needs to be shut off to permit subsequent hepatoblast differentiation[143-148]. Hh signaling is also involved in the maintenance of a hepatic progenitor reservoir throughout life, both in humans and mice. Blockade of Hh activity in hepatic progenitors *in vitro* decreases survival, whereas stimulation of Hh activity inhibits endogenous apoptosis. Both autocrine and paracrine modes of action are suggested, based on simultaneous expression of Hh ligands and receptors in liver progenitor cells[149]. Additional observations suggest that Hh pathway activation is a common feature of various types of chronic liver injury associated with mobilization of hepatic progenitor populations. Participation of the Hh pathway has been demonstrated in the ductular reaction that is elicited by chronic alcohol-induced liver injury in mice and humans[150]; non-alcoholic steatohepatitis in humans[151]; methionine choline-deficient ethionine-supplemented diet[152] and fatty liver damage[153] in mice. Hh ligands are released by hepatocytes and myofibroblasts, and lead to enhanced viability and proliferation of bile ductular cells and hepatic progenitors, thus promoting the ductular response and fibrogenesis. Myofibroblasts, but not hepatocytes, also respond to Hh ligands with an increase in viability[152,154-157]. Either Hh paracrine signaling between myofibroblast and hepatic progenitors or autocrine signaling, or both, promote EMT in hepatic progenitors, which ultimately contributes to liver fibrosis[157-159].

The way in which Hh signaling interacts with other pathways to control the fate of hepatic progenitor populations during liver repair after damage or neoplasia is still not well understood. Nonetheless, some interesting pieces of information are emerging. Similar to that which has been described in embryogenesis[160], a functional crosstalk between the TGF-β and Hh signaling pathways has been proposed in the context of adult liver progenitor expansion during liver injury. In ethanol-fed mice, TGF-β induces production of Hh ligands in hepatocytes,

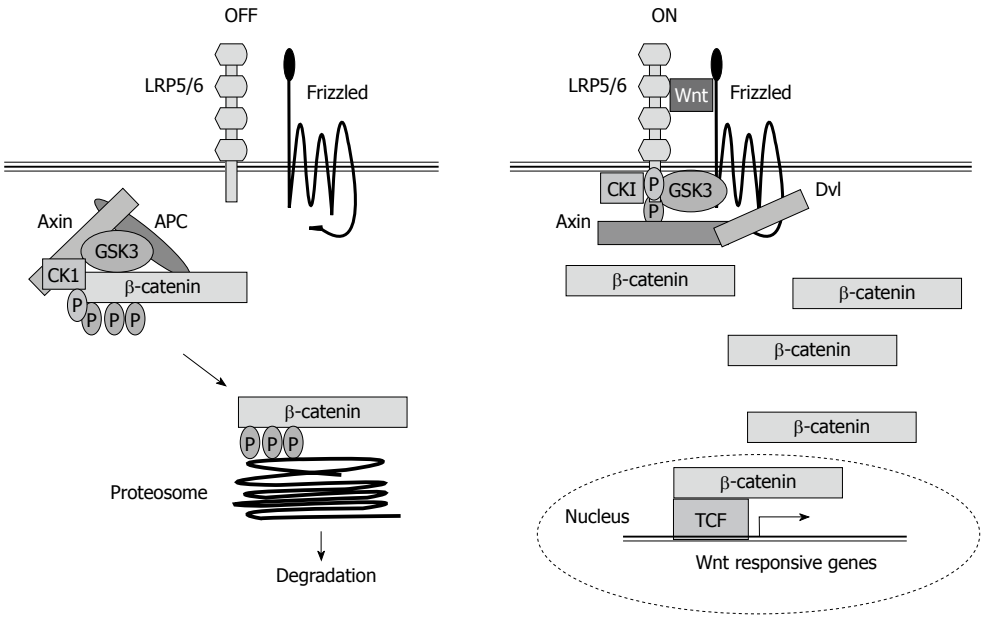

Figure 3 Wnt/β-catenin signaling. Left figure shows how β-catenin is pushed to degradation in the absence of Wnt ligand. Right figure shows how in the presence of Wnt, β-catenin regulates gene expression. TCF: T-cell factor; LRP: Low density lipoprotein receptor-related protein; APC: Adenomatous polyposis coli; CK1: Casein kinase 1; GSK: Glycogen synthase kinase.

which subsequently promotes survival and expansion of ductular/oval cell populations. This supports the concept that enhanced exposure to TGF-β contributes to the accumulation of cells with a ductular phenotype, which are protected from TGF-β-mediated apoptosis[155,161]. Reciprocally, Hh-induced EMT might depend, at least partially, on induction of TGF-β[159]. Further research is required to delineate the Hh-TGF-β interaction.

These findings suggest a role for the Hh pathway as a major mediator in the communication network established between myofibroblast and liver progenitors, which promotes progenitor accumulation and contributes to hepatic fibrosis pathogenesis through EMT induction.

WINGLESS/β-CATENIN PATHWAY

Proteins of the wingless (Wnt) family are secreted signaling molecules that regulate multiple processes in animal development and control tissue homeostasis. The Wnt signaling pathway is highly conserved throughout evolution and plays essential roles in controlling cell proliferation, cell to cell adhesion, and motility[162]. In the liver, it plays many crucial roles during hepatic development and regeneration, and its dysregulation is evident in aberrant growth during hepatocarcinogenesis[163]. Wnt signaling is itself inherently complex[164]. On the one hand, both the ligands and receptors involved in Wnt signal transduction belong to large multi-gene families, which allows for a large number of possible ligand-receptor interactions. On the other hand, Wnt-receptor interactions can elicit a variety of intracellular responses, the best-known of which results in the activation of β-catenin/T-cell factor (TCF) transcriptional complexes. In the absence of signals, β-catenin accumulates in adherens junctions and free β-catenin levels are very low because the tumor suppressor adenomatous polyposis coli forms a trimer complex with glycogen synthase kinase-3-β and axin/conductin, which interacts with and phosphorylates β-catenin, thus targeting it for degradation to the proteasome (Figure 3). Wnts bind a two-part receptor: a seven transmembrane Frizzled and low-density lipoprotein-related protein (LRP)5/6, both being required for canonical signaling. Ligand binding mediates phosphorylation of the cytoplasmic tail of LRP5/6, which creates an axin-binding site. Axin recruitment inactivates the destruction complex. This stabilizes β-catenin, which enters into the nuclei where it displaces Groucho from TCF, nucleating formation of a multiprotein activator complex, and activating Wnt target genes[165] (Figure 3). Furthermore, β-catenin is at the crossroads of growth factor and cytokine signaling. Indeed, certain evidence indicates that TGF-β might induce nuclear β-catenin accumulation, through induction of platelet-derived growth factor signaling[166]. β-catenin expression leads to elevated EGFR levels in hepatocytes and immunohistological analysis shows high correlation between the expression of nuclear/cytoplasmic β-catenin and EGFR in most hepatoblastomas[167]. β-catenin also participates in homotypic cell-cell interactions through its association with E-cadherin. Thus, nuclear β-catenin accumulation in HCC cells might contribute to impaired E-cadherin expression, which mediates the EMT process, migration and survival. It is also known that β-catenin and Met form a complex on hepatocyte membranes. Upon HGF stimulation, the complex is dissociated, in a β-catenin tyrosine phosphorylation-dependent but Wnt-independent manner, which results in β-catenin nuclear translocation[168]. In addition, a role for β-catenin as a downstream effector of HGF in HGF-induced hepatomegaly has been demonstrated[169].

It has been recently suggested that oval cells respond to Wnt ligands *in vitro* with an increase in amino-terminus dephosphorylated β-catenin and cell cycle entry[170], and that canonical Wnt/β-catenin/TCF signaling plays a key role in the normal activation and proliferation of adult hepatic stem cells[171,172]. In this same line of evidence, repression of Wnt/β-catenin signaling in the anterior endoderm is essential for liver and pancreas specification[173], which indicates that turning Wnt signaling off is essential for liver differentiation. An opposite situation might be found during hepatocarcinogenesis, where reactivation of the Wnt/β-catenin pathway, or accumulation of nuclear β-catenin due to other alterations might contribute to the expansion of liver tumor stem cells. Indeed, recent reports have highlighted the relevance of Wnt/β-catenin signaling in the activation of tumorigenic liver progenitor cells[174] and the acquisition of hepatic stem-cell markers[175] in HCC. Accumulation of nuclear β-catenin has been shown to induce an early liver progenitor phenotype in HCC, which correlates with tumor recurrence[176].

In summary, Wnt family and β-catenin/TCF pathways are clearly involved in the maintenance of the liver stemness phenotype, and its repression is necessary for liver differentiation during development. Its overactivation during liver tumorigenesis might contribute to the acquisition of a liver cancer stem cell phenotype.

CONCLUSION

For the past 10-15 years, significant progress has been made in elucidating the cellular and molecular mechanisms that contribute to control of liver stem/progenitor cell behavior. Its complexity and multifaceted nature is evident. Among the diverse signaling pathways at play, the major growth factors, cytokines, and other ligand/receptor systems known to play an important role in liver development, homeostasis and regeneration, certainly occupy a central position. Many of the classical responses elicited by these factors are conserved. However, differences in the downstream mechanisms following ligand-receptor engagement, or signal interaction and crosstalk, as well as specific alterations in intracellular mediators, are being identified as contributing factors to the differential response of liver stem/progenitor cells compared with their mature counterparts. In addition, besides delivery of signals by the surrounding microenvironment, accumulating observations are pointing to co-existence of paracrine and autocrine modes of response as general regulatory mechanisms on this cell population. Collectively, these data indicate that liver stem/progenitor cells follow their own rules and regulations. It is also clear that the same signals that are essential for their activation, expansion, and differentiation, are good candidates to contribute, under adequate conditions, to the paradigm of transformation from a pro-regenerative to a pro-tumorigenic role. Unquestionably, from a clinical perspective, this is a fundamental issue for liver/stem progenitor cell-based therapies. Despite the fact that additional efforts are required to delineate perfectly how the functional switch takes place, the studies summarized here are steps in the right direction.

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EDITORIAL

Paediatric and adult colonic manometry: A tool to help unravel the pathophysiology of constipation

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Abstract

Colonic motility subserves large bowel functions, including absorption, storage, propulsion and defaecation. Colonic motor dysfunction remains the leading hypothesis to explain symptom generation in chronic constipation, a heterogeneous condition which is extremely prevalent in the general population, and has huge socioeconomic impact and individual suffering. Physiological testing plays a crucial role in patient management, as it is now accepted that symptom-based assessment, although important, is unsatisfactory as the sole means of directing therapy. Colonic manometry provides a direct method for studying motor activities of the large bowel, and this review provides a contemporary understanding of how this technique has enhanced our knowledge of normal colonic motor physiology, as well as helping to elucidate pathophysiological mechanisms underlying constipation. Methodological approaches, including available catheter types, placement technique and recording protocols, are covered, along with a detailed description of recorded colonic motor activities. This review also critically examines the role of colonic manometry in current clinical practice, and how manometric assessment may aid diagnosis, classification and guide therapeutic intervention in the constipated individual. Most importantly, this review considers both adult and paediatric patients. Limitations of the procedure and a look to the future are also addressed.

Key words: Colon; Constipation; Manometry; Paediatric; Adult

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INTRODUCTION

Constipation is a common condition, with approximately 15% of adults and 9% of children reporting symptoms[1-4]. The direct and indirect costs are substantial; in the USA alone, an estimated $US1.7 billion/year is spent on adults[5] and a further $US3.9 billion/year on childhood constipation[6]. While anatomical malformation (e.g. anal stenosis, imperforate anus), metabolic and gastrointestinal causes (e.g. hypothyroidism, celiac disease, and cystic fibrosis),

intestinal nerve or muscle disorders (e.g. Hirschsprung disease, chronic idiopathic intestinal pseudo-obstruction), childbirth or pelvic surgery are known to cause constipation, for many patients, their condition is regarded as "idiopathic". Indeed, in children, it has been reported that less than 10% of those suffering from constipation have a recognizable underlying organic cause[7,8]. Defects in the nerves and pacemaker cells (e.g. decreased ICC density[9]) could be responsible in a proportion of cases, but as yet, techniques are not sufficiently developed to clearly identify such altered morphology, rendering any attribution to patho-aetiological significance (cause, effect, or epiphenomenon) speculative[8,10,11].

Given that both aetiology and pathophysiology are unclear in many cases, it is perhaps therefore not surprising that up to one-third of adults and children who seek medical help will fail non-surgical therapy[12,13]. Patients refractory to such interventions are debilitated, with physical functioning, mental health, general health and bodily pain all scoring poorly on quality of life questionnaires, compared to the non-constipated population[14]; indeed, the impact is comparable to other chronic conditions, such as inflammatory bowel disease in adults[15] and cancer in children[16]. In refractory cases, surgery, including subtotal or total colectomy, repair of anatomical anomalies believed to impede defaecation[17,18], appendicostomies[19], and more recently sacral nerve stimulation[20] may be considered. However surgical procedures are not without risk, with reported post-operative morbidity often high, and functional outcomes suboptimal[17,21,22]; this, in turn, equates to poor quality of life[23].

Improving therapies for functional bowel disorders is a fundamental goal for all researchers and clinicians working in this field, and to achieve this objective, a better understanding of pathophysiologies that may underpin such disorders is essential. In chronic constipation, altered colonic motor function remains the leading hypothesis to explain symptom generation, and this may be true not only for constipation associated with delayed colonic transit[24], but also for constipation allied to an evacuatory disorder[25]. In clinical practice, routine investigation of colonic motor function is achieved by use of transit studies (typically radio-opaque markers[24,26]), while defecatory function typically is assessed using evacuation proctography or balloon expulsion. Diagnostic manometry, which provides a direct assessment of those contractile activities subserving intestinal functions, is well developed for other parts of the gastrointestinal (GI) tract, notably the oesophagus and anorectum, but its diagnostic potential in the colon remains less certain. This is due to a number of factors including the relative inaccessibility of the proximal large bowel, the technical constrains of current manometric recording systems, and inadequate understanding of normal colonic motor physiology, which is fundamental to confidently diagnosing motor abnormalities in disease states. This review examines the equipment and protocols used to record colonic motor patterns *in vivo* in humans, with particular focus on the diagnostic relevance of data obtained, and the ability of colonic manometry to guide treatment. Current limitations and future direction for the procedure are also covered.

COLONIC MANOMETRY

Manometric studies of true colonic (as opposed to recto-sigmoid) motor function have been reported upon since the late 1980s[27]. However, the colon remains the least well understood section of the GI tract, particularly its proximal portions. For constipation, around 20 studies in adults, and fewer in paediatric populations, have been published. As such, colonic manometry is still largely used as a research tool in adults, with relatively few authors advocating diagnostic use[28,29]. Nevertheless, a recent American Neurogastroenterology and Motility Society consensus statement has recommended this test for the assessment of severely constipated patients (both adult and paediatric) who are unresponsive to medical therapy, and who have evidence of slow colonic transit in the absence of an evacuatory disorder[30]. Furthermore, in children, colonic manometry has been proposed to discriminate normal colonic motility from colonic neuromuscular disorders[31]. It may also help to clarify the pathophysiology of persistent lower GI symptoms after surgery for Hirschsprung disease, evaluate colonic involvement in a child diagnosed with idiopathic intestinal pseudo-obstruction syndrome, assess function of a diverted colon prior to possible re-anastomosis, and to assess colonic motor activity prior to intestinal transplantation to determine if the colon should be kept at the time of transplant[32]. What is certainly true is that colonic manometric procedures lack standardisation, with a variety of catheter types, placement techniques and protocols currently used.

It must also be remembered that colonic manometry is an invasive procedure, and as such should only be performed in those patients with symptoms severe enough to have a marked impact on their quality of life and social well-being. Extensive conventional treatment should be offered before testing colonic motility.

Catheter types

In general, there are two broad types of catheters used: (1) water-perfused; and (2) solid-state. As yet, there have been no studies which directly compare the two types. Preference for one over the other is guided by existing equipment, desired application, study design, and particularly cost.

Water-perfused manometry catheters: Water-perfused manometry is used in children and has been the preferred choice in the vast majority of studies in adults. Catheters are made of flexible PVC[27,33] or extruded lengths of silicone rubber[34]. The latter is highly flexible, and is desirable for patient tolerability if an oral route is used for intubation (see below). The catheters incorporate between 4 and 16 recording ports or side-holes, with an inter-side-hole distance of between 1 and 15 cm[24,35]. Each recording port

consists of an open lumen, which is connected to a pneumohydraulic infusion pump that ensures constant flow of water. The basic premise for recording is that contractions of the colonic wall occlude the manometric ports, thus impeding flow of perfusate (degassed water). Resistance to flow is transmitted as pressure change to external transducers, and the degree of resistance depends upon the amplitude and duration of the contraction. Such catheters are relatively inexpensive and highly versatile with regard to configuration (number, position and orientation of side-holes); in addition, silicone catheters are autoclaveable, and therefore can be re-used many times without fear of contamination. However, due to the requirement of being attached to the manometry system, and need for constant water infusion, studies are always performed in the laboratory setting. This confines the subject to a bed for the length of the recording period with minimal mobility allowed, thus creating a somewhat unphysiological environment. In addition, the volume of water introduced into the lumen is considerable (> 3 litres) if recording from multiple side-holes for prolonged periods of time. Whilst the colon does have the ability to absorb up to 6 litres of fluid a day[36], the effects of introduction of this amount of fluid upon colonic motility remains unknown.

Solid state manometry catheters: So-called 'solid-state' assemblies traditionally consist of strain gauges embedded into a flexible PVC catheter. Each strain gauge is attached to an amplification/recording system *via* fine connective wiring[35]. Until recently, solid-state catheters have typically had between 6 and 10 sensors spaced at 7-15 cm intervals[24]; however, recent technological advances now allow in excess of 20 sensors. Recorded pressure signals can be stored on portable digital recorders enabling the subject to be ambulant[29,37]. However, the cost of these catheters is considerable in comparison to water-perfused catheters, and there has been a tendency in the past for recording channels to break[38,39]. Nevertheless, with further technological improvements, and recent developments in fibre-optic technology, which has seen the emergence of an optical manometry system (see Future directions below), such limitations should be overcome[40,41].

Placement techniques

In human subjects, gaining access to the colon can generally be achieved through two routes: *antegrade* placement *via* the nose or mouth, or *retrograde* placement *via* the anus. Colonic catheters can alternatively be placed through a variety of stomas, a method used particularly in children[42-44] but also in adults[45].

Retrograde placement: This provides the easiest access to the colon, and is by far the most commonly used procedure in both adults and children[33,37,46,47]. In adults, catheters are placed with the aid of a colonoscope and can be positioned in the desired location. To date, most studies utilising this method have located the tip of the catheter at the hepatic flexure or mid-transverse colon, meaning that recordings are only achieved from sites distal to this. However, the Sydney group have achieved true pancolonic recordings over the past few years by securing the tip of the catheter to ascending colonic/caecal mucosal folds using endoclips[20,29,48,49]; this has the advantage of helping to prevent catheter displacement or excretion during defecation[50,51].

In children, the retrograde placement of colonic manometry catheters has also utilised colonoscopic procedures[46]; however, in recent years colonic catheters have also been successfully placed using fluoroscopic guidance alone, both through stomas and *via* the rectum[44]. However, this technique may be associated with prolonged exposure to radiation, in some cases up to 27 min[44].

One of the major advantages of retrograde catheter intubation is subject tolerability. Once the catheter has been sited, there is little or no discomfort. However, in addition to concerns over catheter displacement, there are other potential disadvantages, most notably the requirement for bowel preparation and usually some form of anaesthesia or sedation. The removal of faeces from the colon has recently been shown to result in an increase in the frequency of high amplitude propagating contractions[52,53], and also to disrupt the spatiotemporal organisation of propagating sequences (PSs)[53]. However, the colonic responses to physiological stimuli, such as a meal and morning waking, remain unchanged[53]. Nevertheless, it could be argued that bowel preparation is advantageous, in that the 'starting point' for each study, provided that bowel preparation is performed in an equivalent manner, is standardised.

Antegrade placement: This is a less common method for recording colonic motility and is restricted to adult studies[25,34,39,54]. The catheter is fed through the nasal cavity or mouth, and once through the pylorus (often achieved under fluoroscopic guidance), a balloon on the tip of the catheter is inflated with air or water to facilitate transit through the small and large bowel[25,34]. The main advantage of this procedure is that it obviates the need for bowel preparation, and thus the study is performed under essentially normal basal physiological conditions. The procedure is, however, time-consuming in comparison to retrograde catheter placement. Even in healthy controls, it may take up to 36 h to intubate the distal descending colon[34,35]. Furthermore, with the catheter *in situ* for a prolonged period of time, subject tolerability becomes an issue with nasal/oral/pharyngeal discomfort and nausea commonly reported. Finally, while antegrade placement can be used to study colonic motility in health and in patients with relatively normal colonic transit[25], its use in patients with slow transit constipation (STC) is problematic. As antegrade catheter placement relies on peristalsis to promote the catheter tip through the gut, the process can be greatly prolonged in patients with a disorder characterised by delayed gut transit (the primary clinical indication for this investigation[30]), especially when small bowel motility may also be also abnormal[55].

Placement through a stoma: Intubation *via* appendicostomy, caecostomy, ileostomy or colostomy has been used to successfully investigate children with STC[19,44,56]. In such subjects, after a preceding colonic washout, the in-dwelling device (e.g. Chait caecostomy button) is removed. Two techniques can then be adapted: (1) following sedation, the catheter can be advanced through the stoma with the aid of a guidewire, and positioned under fluoroscopic guidance[44]; and (2) a 10F feeding tube can be positioned through the stoma through which a Bisacodyl solution (2-4 mg) can be instilled directly into the caecum. A manometry catheter can then be introduced through the feeding tube and advanced in an antegrade direction along the colon. The Bisacodyl induces propulsive activity, and a balloon on the tip of the catheter can be inflated with water to aid propulsion through the large bowel[42,43].

RECORDED COLONIC MOTOR ACTIVITIES

Non-propagating motor activity

Non-propagating activity makes up the majority of a colonic manometric recording, consisting of apparently random pressure waves recorded at single or multiple recording sites, often alternating with phases of motor quiescence. Pressure waves may be sub-classified on the basis of duration, and are considered to be of either long duration (the majority) or short duration (often superimposed on long duration pressure waves). The presence of two types of phasic contraction in the colon is unique, compared with the remainder of the GI tract[57]. The functionality of non-propagating activity is outlined below.

Propagating motor activity

Propagating pressure waves are categorised into PSs (also termed propagating contractions) and high amplitude PSs (HAPSs; also termed high amplitude propagating contractions; Figure 1); the latter are recognised as the motor correlate of mass intraluminal movement and are involved in defaecation (Figure 2)[59-62]. Both PSs and HAPSs can be further qualified by the terms antegrade (aboral) or retrograde (oral), depending upon the direction of propagation. However, criteria used to define these events are inconsistent[35]. A general classification includes an array of 3 or more pressure waves recorded from adjacent recording sites, with a trough to peak amplitude $\geqslant$ 5 mmHg per pressure wave, in which the conduction velocity between wave onsets is between 0.2 and 12 cm/s[34,50]. The definition of what constitutes a HAPS is based on a threshold value for amplitude of one or more of the component pressure waves[35]; this varies markedly in the literature from > 50 to 136 mmHg[34,50,63-67]. Consequently, the range of published frequencies is wide[35]; in many cases, the chosen threshold value appears to be arbitrary. Some, however, are based on proven functional characteristics[34].

PSs are identified throughout the large bowel, although they originate with greater frequency in the proximal compared to the distal colon[37,38,58,59,68,69]. The majority of ascending colonic PSs do not migrate beyond the splenic flexure, and conduction velocity of PSs increases as the PS moves distally from the proximal colon, as does the amplitude of the propagating pressure waves[50,58,59,70].

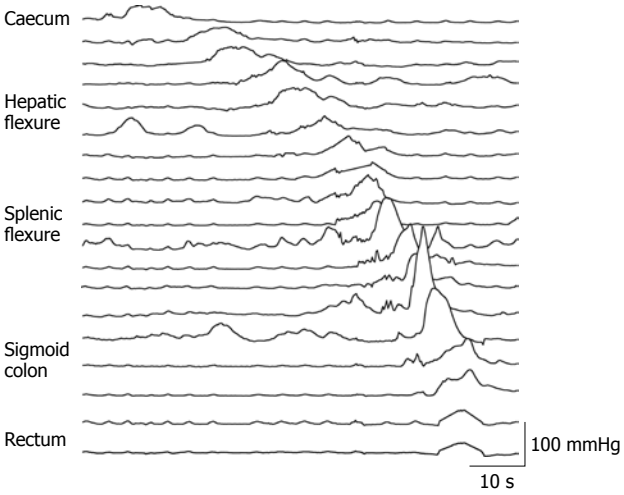

Figure 1 The high amplitude propagating sequence was first described in 1988[27]. The majority of these propagating events originate in the proximal colon and extend to the sigmoid colon. The amplitude of the component pressure waves increase as the motor patterns reaches the descending colon[58].

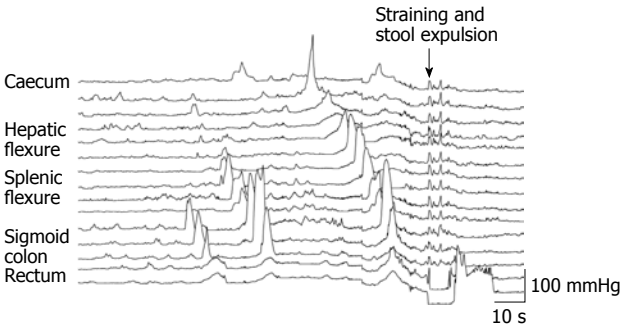

Figure 2 High amplitude propagating sequences are commonly associated with defaecation in healthy controls. Note the series of three high amplitude propagating sequences (HAPSs) prior to stool expulsion. The first originates at the distal descending colon, and with each subsequent HAPS, the site of origin moves to a more proximal location with the final HAPS extending the entire length of the colon[34].

Given their functional significance, HAPSs have garnered most interest in terms of pathological implication, and indeed decreased HAPS frequency is widely accepted as one of the principal hallmarks of STC (see below[27,29]). However, focus on propagated pressure waves alone, as is the case in many published reports, has perhaps led to their clinical impact being over-stressed, particularly given that they occur only a minority of the time during a colonic manometric recording; the organisation of other, more common motor activities may be of similar (or even greater) importance.

Organised colonic motor patterns

Akin to the interdigestive migrating motor complex of the upper GI tract[71], the colon exhibits periodic bursts of regular phasic pressure wave activity, termed either colonic

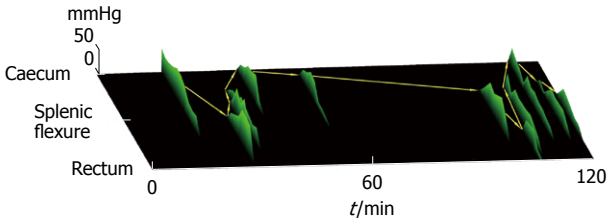

Figure 3 Two hour spatiotemporal maps of colonic propagating sequences in a female healthy control. In this map each individual ridge represents an antegrade propagating sequence. The start of each ridge indicates the site of origin and the time of day the propagating sequence occurred. The length of the ridge indicates the extent of propagation. The heights of the ridge indicate the amplitude of the component pressure waves. The yellow arrows link the site of origin of sequential propagating sequences. While no single propagating sequence spans the entire colon collectively, a linked series of propagating sequences can do so[76].

motor complexes[38,39,58] or rectal motor complexes (MC)[35,72], depending upon their site of origin. MCs predominate in the sigmoid or rectum, where they have also been termed "periodic rectal motor activity"[37,73,74]. The functional significance of these events is unclear, although it has been suggested that they provide a "brake" around the rectosigmoid junction[73], and reflect enteric neuromotor integrity, given that they occur in extrinsically denervated colon preparations[75].

In addition to MCs, sequential PSs have been shown to be linked in organized spatiotemporal patterns throughout the colon[76]. Many of these PSs form series in which three or more consecutive PSs demonstrate a regional shift in the colonic region from which they originate, i.e. each PS in a linked series will originate in a progressively more proximal or distal colonic location. Whilst most single PSs do not span the length the colon, collectively, a series of linked PSs can do so. It is likely that such linkage is important for the transport of content over longer lengths of the colon (Figure 3)[76].

COLONIC MANOMETRY PROTOCOLS

Paediatric studies

Despite the variety of placement techniques and catheter types, once the catheter is positioned, there is commonality to the actual recording protocol. In children, clinical studies generally do not last more than 4-5 h[77], but 24 h studies have been used in the research setting to provide more detailed information on motility characteristics[42,43]. Paediatric colonic manometry catheters typically contain 6-8 recording side holes, evenly spaced at 7.5, 10 or 15 cm intervals[42,43,77]. The paediatric colonic motility test includes at least 1 h of fasting and 1 h postprandial after finishing a high calorie meal. During the test, the child is required to remain in bed and it is important to have a trained observer at the bedside. If HAPSs are not recorded during the fasted or postprandial period, then drug stimulation with Bisacodyl (0.2 mg/kg, max 10 mg, diluted with 0.9% NaCl) is administered. HAPSs induced by Bisacodyl usually occur within a few minutes and are identical to spontaneous HAPSs. A cramping sensation and the urge to defaecate often accompany the presence of HAPSs[47]. If the paediatric test extends to 24 h, then the nocturnal frequency of motor patterns and the colonic response to waking are also measured[42,43,77].

Adult studies

In published adult colonic manometry studies, similar protocols are followed, although the catheter length is generally longer, containing up 16 sensors (water-perfused) spaced at 7.5 cm intervals[25,34]. Recordings are typically of 24 h duration. All studies examine two or more of the following: (1) fasting; (2) meal responses; (3) nocturnal suppression; (4) morning waking; and (5) response to chemical stimuli[24,35].

Normative data

For a clinical diagnosis, data collected from patients is compared to that obtained from healthy controls. In the adult population, recruitment of volunteers is generally not problematic, allowing normal ranges to be developed for various parameters of the motility recording (though it must again be stated that the size of published normative data sets remains inadequate).

By contrast, colonic motility recordings in truly healthy paediatric control subjects are lacking for obvious ethical reasons. Di Lorenzo *et al*[78] attempted colonic manometry in 32 "healthy" paediatric patients, though they were not strictly healthy controls, as they had diagnoses ranging from functional faecal retention (n = 15) and non-ulcer dyspepsia (n = 10), to Munchausen by proxy (n = 7). Other paediatric studies in adolescents with STC have used healthy young adults as the control group; whether this is appropriate is unknown[42,43].

OBSERVATIONS FROM COLONIC MANOMETRY STUDIES

Both propagating and non-propagating motor patterns may be temporally associated with propulsion and mixing of colonic content[59,60,79], signifying that these are important determinants of normal physiology in health and of pathophysiology in patient groups. In adult and paediatric patients with chronic constipation, a number of findings have been made which suggest pathological significance, including: (1) a lack of the normal[37,58,80] increase in colonic motor activity after a high calorie meal[29,43,46,49,80-86] (Figure 4); (2) a lack of the normal[29,50,51,58] suppression of colonic motor activity at night, and a suppressed or absent increase in colonic activity in response to morning waking[25,29,43,80] (Figure 4); (3) a decrease in frequency of HAPSs[27-29,39,47,49,80,83,85-88], although normal daily frequencies have also been reported in some constipated subjects[25,43] (Figure 4); (4) increased non-propagating activity in the left colon[89]; (5) absence[25] of the characteristic spatiotemporal patterning of PSs preceding normal spontaneous defecation[34]; (6) diminished co-ordination between

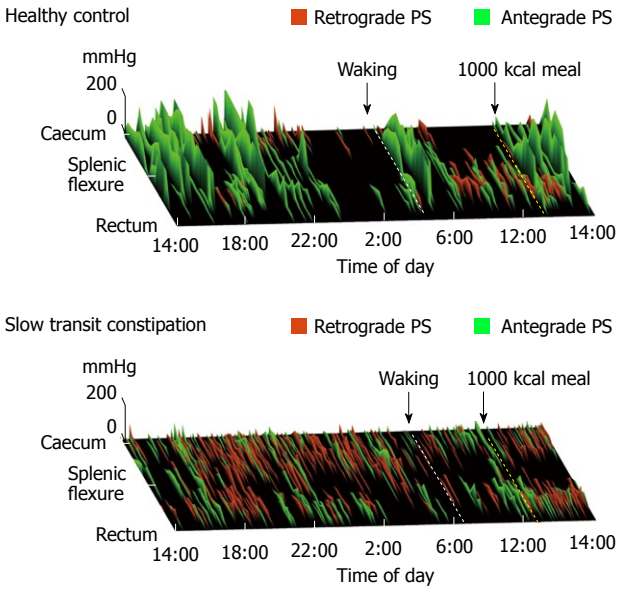

Figure 4 Twenty-four hour spatiotemporal maps[80] of colonic propagating sequences in a female healthy control and a female patient with slow transit constipation. Within each map the ridges represent antegrade (green) and retrograde (red) propagating sequences (PS). The antegrade PSs originate at the orad end of the green ridge and retrograde PSs originate at the anal end of the red ridges. The start of each antegrade and retrograde ridge indicates the site of origin and the time of day the PS occurred. The length of the ridge indicates the extent of propagation. The height of the ridge indicates the amplitude of the component pressure waves. The yellow-hatched line indicates when the 1000 kcal lunch was given, the white-hatched line indicates the time of morning waking. In health the frequency of propagating sequences are reduced during sleep and increase upon morning waking and in response to a high calorie meal. The map in the STC patients is characterized by an increased frequency of short extent PSs and a lack of high amplitude propagating sequences. There is an absent meal response (no increase in PSs) and an absent nocturnal suppression of PSs.

sequential pan-colonic PSs[76,80]; (7) a blunting of the normal[49] increase in PS or HAPS frequency in response to rectal or colonic infusion of Bisacodyl has been shown in a proportion of individuals[47,49,85], although in the majority of patients a normal response is recorded[49,83,85,90-92]; (8) a blunting of the normal increase in colonic HAPS frequency to intravenous injection of the cholinergic agonist edrophonium chloride[92]; (9) a blunting of the normal proximal colonic increase in motor activity in response to a rectal infusion of chenodeoxycholic acid[93]; (10) an increase in the frequency of HAPSs in response to sacral nerve stimulation in patients with STC[20]; and (11) a significant increase in the frequency of rectosigmoid motor complex activity[73,74].

CAN COLONIC MANOMETRY FINDINGS HELP TO DISTINGUISH SUBTYPES OF CONSTIPATION?

Constipation can be conceptualized into two broad overlapping categories: STC, and disorders of evacuation[1,94-96]. In some cases, this distinction among subgroups, based on aberrant physiological measurement, has proven beneficial in planning treatment and in predicting therapeutic outcome[96-99]. For example, identification of dyssynergic defaecation by anorectal manometry and balloon expulsion testing, in the absence of delayed colonic transit, is predictive of a high success rate from biofeedback[97,98]. Likewise, in carefully selected patients with severe STC, where the transit delay is restricted to the colon[17,22,100,101], and there is no evidence of evacuatory dysfunction, surgery such as colectomy with ileorectal anastomosis may be effective. In children this group is successfully managed with an appendix stoma and antegrade enemas[19].

In terms of colonic manometric investigation, specific biomarkers or biosignatures that may help to define constipation subtypes (and thus provide therapeutic targets) have, as yet, not been fully established. In adults, only three studies have addressed the validity of manometric findings between "recognised" subgroups[102-104]. The first concluded that intraluminal measurements could not be used to discriminate between subgroups of patients with either STC or an evacuation disorder[102]. The second study examined three subgroups of constipated patients with either STC, an evacuation disorder, or 'normal transit' constipation, and found that fasting and postprandial motor parameters were not useful for discriminating amongst these subtypes, although colonic compliance was[103]. Bassotti *et al*[104] noted that no colonic motor patterns were able to differentiate between constipated patients with or without delayed transit.

A further study in patients with constipation secondary to antidepressants revealed minimal motility differences compared to patients with idiopathic constipation[85]. Finally, Hervé *et al*[83] took the approach that manometric findings themselves should be used as the basis for subclassification. In a study of 40 adults with STC, they identified 4 subgroups: group 1 displayed no HAPS or a colonic response to a high calorie meal; group 2 showed increased sigmoid motor activity; group 3 had a reduced frequency of HAPSs; and group 4 displayed normal colonic motility. However the clinical impact of such findings, in terms of guiding successful management, remained undetermined. Nevertheless, through sufficiently large scale studies, a classification system incorporating findings from pancolonic manometric investigations, rather than being based on traditional studies of colonic transit and evacuatory function alone, may be merited. Current stratification, where the colon is seen to be responsible for transit, and the anorectum solely responsible for evacuation, is likely a gross oversimplification.

CAN COLONIC MANOMETRY HELP TO GUIDE TREATMENT AND IMPROVE OUTCOMES?

The primary indicators of abnormal colonic motor function in constipation (see above) are reduced HAPS frequency and a diminished or absent response to eating, morning waking or chemical stimulation. The colonic re-

sponse to ingestion of a meal is likely to involve the CNS and neurohormonal pathways; a suppressed response has been proposed as indicative of colonic myopathy and an absent response as a possible neuropathy[29]. Similarly, diurnal variation in colonic motor activity is likely to be mediated by the CNS, and therefore a diminished or absent response to sleep or morning waking supports a neuropathic cause in such patients[29]. With regard to chemical stimuli, a failed response may indicate an abnormality within the myenteric plexus[105], cholinergic pathways[92] or recto-colonic neural pathways[93].

Adult studies

In adults, very few interventional studies have been based upon evidence gained from colonic manometric investigation. In 3 patients with severe constipation who had failed conservative therapy, Bassotti *et al*[28] demonstrated globally reduced colonic motility, a minimal meal response and no response to Edrophonium. Based upon these data, 2 patients underwent a total colectomy and 1 a hemicolectomy, with "fairly good results at follow-up" reported. No long-term data were provided.

In a larger study, Rao *et al*[29] performed 24 h colonic manometry in 21 patients with STC. Utilising the response to ingestion of a meal, response to morning waking, and HAPS frequency as diagnostic criteria, patients were classified as having a neuropathy (n = 10) if they had an absence of two or more, a myopathy (n = 5) if they had a reduced response to two or more, or being normal (n = 6). Those with a suspected neuropathy were offered a colectomy (performed in 7/10, with "improved" bowel symptoms reported; no follow-up duration specified). Those with a myopathy were offered a regime of biofeedback and laxatives, and reported "modest improvement" at 1 year.

Paediatric studies

In children, colonic manometry studies are far more likely to guide treatment options. In a study by Pensabene *et al*[106], results of colonic manometry testing resulted in a recommendation to change therapy (mostly surgical) in 93% of the patients with intractable organic or functional constipation. Importantly, 88% of the 98 parents believed that the therapeutic suggestions made had been helpful in improving their child's health[106].

Di Lorenzo *et al*[107] studied 46 children with constipation and/or faecal incontinence after surgery for Hirschsprung disease. Four groups were established based upon results from colonic manometry: (1) HAPSs propagating from the proximal colon through the neorectum to the anal sphincter, which was associated with faecal incontinence (n = 18); (2) an absence of HAPSs and lack of colonic meal response, which was predominantly associated with constipation (n = 15); (3) a normal manometric study, with a hypertensive anal sphincter, which was invariably associated with constipation (n = 4); and (4) a normal manometric study, but the child presented with fear of defaecation and retentive posturing, which was predominantly associated with constipation and soiling (n = 9). Those in group one were treated with anticholinergics and Loperamide. In group two, resection of the abnormal section of colon was recommended. In the third group, four-quadrant intrasphincteric botulinum toxin injections were recommended, and in the fourth group, a programme of behaviour modification and stool softeners were used[107]. Based upon these treatments, the authors reported an improvement in global health and emotional health scores as well as an improvement in the number of bowel movements and resolution of abdominal pain[107].

Also in paediatric patients, colonic manometry is reported to have value in determining poor outcomes for the surgical treatment of intractable constipation using an appendicostomy or a caecostomy with antegrade irrigation of the colon[47]. For example, those children with a poor outcome also displayed an absence of colonic response to Bisacodyl. However, the fact that 50% of children with no production of HAPS still had good outcomes to antegrade irrigation suggests that the Bisacodyl response is by no means a solid predictor[47].

CURRENT LIMITATIONS

While colonic manometry studies in both children and adults have helped to define some of the physiological and pathophysiological aspects of colonic motor function, there are still no published quantitative data on propagating or non-propagating activity that clearly differentiates healthy subjects from constipated patients[30]. Furthermore, at least in children, morphological changes reflecting possible myogenic or neurogenic disorders in muscle tissue do not correlate with particular features of colonic manometric recordings[108] or anorectal manometry[109]. It also remains unclear whether data derived from adult studies can be extrapolated to children and *vice versa*. No manometric studies using the same equipment and protocols have compared childhood and adult constipation. Indeed, because a normal frequency of HAPSs has been identified in children with STC (contradicting most reported findings in adults), childhood constipation may represent a different entity[43]. The impact of sensory, as opposed to motor dysfunction on the development of constipation is gaining increasing recognition[110,111]. Nevertheless, the two are inextricably linked, and future studies should look to explore both domains concurrently, by the best methods available, rather than consider certain functional bowel conditions as principally motor disorders (e.g. constipation), and others as sensory disorders (e.g. the irritable bowel syndrome).

With variations in protocols, catheter types, numbers of recording sites, spacing between recording sites, placement techniques and definitions of recorded activities, it is perhaps not surprising that consistent findings have not been reported in the literature[24,35]. Furthermore (and as stated above[24]), almost all studies that detail "colonic" manometry have, in reality, recorded colonic motor patterns distal to the mid-transverse colon. Support for the use of true pancolonic manometry resides in the fact that

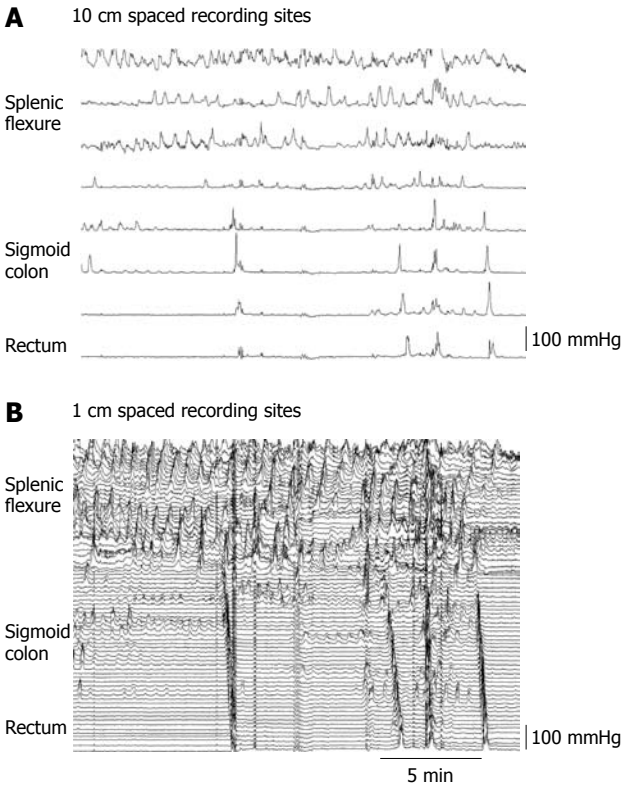

Figure 5 Manometric traces of colonic motor activity recorded by a fibre-optic manometry catheter with 90 sensors spaced at 1 cm intervals. A: To represent common sensor spacing for current published colonic recordings, the data set, obtained through "high resolution" manometry, is sub-sampled to present every 10th channel (10 cm spacing, top); B: The complete data set, captured at 1 cm intervals, is displayed at the bottom. Note that at 10 cm spacing, the most proximal three channels reveal what appears to be non-propagating pressure waves. However, when the full data set is viewed (B), a series of short-extent retrograde propagating events are detected. These events are missed when spatial resolution is poor. Modified from[41].

PS and HAPS are not distributed evenly throughout the colon, and predominantly originate in the ascending and proximal transverse colon, and that the entire colon appears to function in a coordinated fashion[76,112]. 'Partial' colonic recordings thus provide an incomplete picture of colonic (patho)physiology.

FUTURE DIRECTIONS

In the oesophagus and the anorectum, manometry is an established diagnostic tool. In comparison to the colon, these regions afford easier access and therefore have been subjected to far more research and clinical investigation. More recently, development of high resolution manometry (HRM) has further enhanced diagnostic potential, particularly in the oesophagus[113], most notably with regard to standardisation of recording procedures. With the advent of fibre-optic manometry[40,114], HRM recordings are now feasible throughout the entire colon. Catheters incorporating up to 120 sensors spaced at 1 cm intervals have already demonstrated that the vast majority of PSs propagating over short distances are missed by recording sites spaced > 7 cm apart[40,41] (Figure 5). If such technology is able to identify those elusive biomarkers that can help to reliably distinguish constipation subtypes and guide management, then this provides the opportunity to take colonic manometry out of the research arena and into clinical practice. Such progress is only possible through appropriately designed and powered studies, utilising equivalent hardware and software, both in adults and children. The revolution that has been seen with oesophageal HRM, such that traditional manometry is now regarded as obsolete, can serve as a template for the clinical potential of pancolonic manometric investigation.

CONCLUSION

Pancolonic manometric investigation provides a unique window onto motor functions of the large bowel, and through its use we now have a greater understanding of normal colonic physiology and also the pathophysiology of constipation in both adults and children. Although a recent consensus statement[30] advocates use of colonic manometry in patients with "significant motility disorders", the technique is still not refined enough for widespread clinical use, and no prospective and controlled studies have been performed evaluating the clinical value of this tool. Nevertheless, technological advances offer the potential of adding colonic manometry to the available diagnostic armamentarium in broader clinical practice. To achieve such a goal, there is a fundamental requirement for standardisation of methodologies and recording protocols, further development of automated analysis software, and a vital need for generating larger normative data sets, which will enable diagnostic yield to improve. This will be achieved through the coordinated efforts of those dedicated to this field. Further clinical exploration of this technique affords the possibility of a more directed therapeutic approach in patients with chronic constipation. Colonic manometric studies, performed in large numbers of constipated patients, will hopefully establish biomarkers that can distinguish existing (largely predetermined) constipation subtypes (i.e. STC, evacuation disorders), or aid a more contemporary phenotypic classification.

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TOPIC HIGHLIGHT

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Transient elastography in chronic hepatitis B: An Asian perspective

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Abstract

Transient elastography (TE) is a new non-invasive tool for assessing liver stiffness, which is correlated with the histologic stage of liver fibrosis. Many studies have reported a good accuracy of TE in predicting significant fibrosis and an optimal accuracy in predicting cirrhosis. Furthermore, the potential role of TE in screening the general population has also been proven. TE thus helps physicians to decide treatment strategies, predict prognosis, and monitor disease progression in patients with chronic liver disease and to screen the general population to identify high risk patients with potential liver disease. However, most data on the clinical roles of TE have been gathered in European patients with chronic hepatitis C (CHC), because TE was first developed in France. Accordingly, much data on the usefulness of TE in patients with CHC has accumulated. Recently, however, vigorous efforts have been made to apply TE to patients with chronic hepatitis B (CHB), and TE has also proved to have acceptable accuracy in diagnosing liver fibrosis and cirrhosis in these patients. Thus, we focused on TE in the Asian population with CHB in comparison with the European population with CHC and found that the diagnostic performance and cutoff values were different between the 2 populations possibly as a result of several different confounders between Asian and European populations (the etiology of chronic liver disease, histologic features, major fluctuation in alanine aminotransferase levels, and the prevalence of high body mass index and metabolic syndrome). Therefore, further studies tailored to the Asian population with CHB should be performed before the widespread application of TE in Asian populations with CHB.

Key words: Asia; Chronic hepatitis B; Fibroscan; Hepatitis B virus; Liver stiffness measurement; Transient elastography

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INTRODUCTION

Transient elastography (TE) using FibroScan® (EchoSens, Paris, France) is a newly introduced non-invasive tool, that generates an elastic wave using a vibrator applied to the thoracic wall at the level of the right lobe of the liver and measures the propagation velocity of the wave,

which is directly associated with liver stiffness (LS)[1]. To date, the clinical utility of TE has been widely reviewed, and it is regarded as having considerable accuracy for predicting liver cirrhosis in patients with chronic liver disease (CLD) of diverse etiologies[2-5]. TE is quick, highly reproducible, and completely harmless to patients. In addition, it can be learned and performed easily after a short training period (by nurses or technicians) without consuming the time of clinicians. For these reasons, TE has become popular in clinical practice as a tool for aiding in the diagnosis and follow-up of liver disease.

Because TE was first developed in France, most studies on its benefits have been explored in European countries where chronic hepatitis C (CHC) is prevalent. Accordingly, extensive data on the clinical roles of TE in assessing liver fibrosis in patients with CHC have been gathered. Recently, several meta-analysis studies reported that TE is a reliable non-invasive tool to detect advanced liver fibrosis and liver cirrhosis[6-8]. However, most studies included in the meta-analysis investigated European populations with CHC. Data from Europe cannot be extrapolated to the Asian population, as subsequent trials of TE in Asian countries where hepatitis B virus (HBV) infection is more prevalent than hepatitis C virus (HCV), displayed divergent results. In this Topic Highlight, we will focus on TE in the Asian population with chronic hepatitis B (CHB).

METHODOLOGICAL CONSIDERATION OF TE IN THE ASIAN POPULATION

LS values are defined as the median of 10 valid measurements and at least 60% of TE shots should be successful for each examination according to the manufacturer's recommendations. Accordingly, to date, only the results of TE examinations satisfying the above criteria have been considered as reliable and have been included for the analysis in reported studies.

Another important parameter for confirming the validity of LS values is the interquartile range (IQR), which is defined as the values between which 50% of observations fall. The lower boundary is the 25th percentile (lower quartile), the upper boundary is the 75th percentile (upper quartile). Generally, the IQR/median LS value ratio (IQR/M) should not exceed 30% if the reliability of LS values is to be preserved. However, recently, Lucidarme *et al*[9] proposed new criteria for reliable LS values, using the IQR/M based on the data from more than 250 French patients with CHC. They found, in a multivariate analysis, the fibrosis stage (F0-2 *vs* F3-4) and IQR/M were significantly associated with significant discordances between TE and liver biopsy (LB), with an optimal discriminant cutoff value of 0.21. They thus concluded that the IQR/M can overestimate liver fibrosis using TE, irrespective of success rate, when the significance discordance was defined as a discordance of at least 2 stages of fibrosis between LS values by TE examination and the METAVIR scoring system. From these results, they suggested a novel algorithm for clinical practice as first-line assessment of liver fibrosis in patients with CHC (Figure 1).

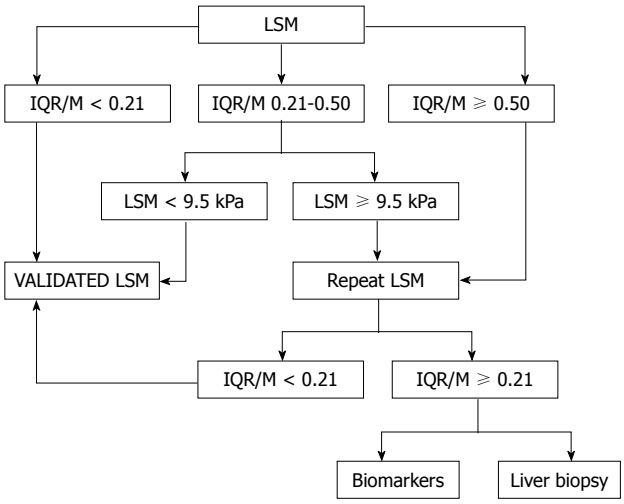

Figure 1 Suggested algorithm for clinical practice as first-line assessment of hepatic fibrosis in patients with chronic hepatitis C. IQR/M: Interquartile range/median liver stiffness value ratio; LSM: Liver stiffness measurement.

Because the IQR/M was confirmed in only one European study with CHC showing relatively high body mass index (BMI) (25 ± 4.1 kg/m^2), its application to the Asian population with CHB (who generally show a lower BMI) is difficult and requires further validation. Although the data are preliminary, we have attempted to validate the IQR/M for our cohort of 156 patients with CHB (mean age 38.8 years, male 75.0%, mean BMI 23.2 kg/m^2) who underwent LB and TE prior to starting antiviral treatment. In this study, we selected the cutoff LS values of Marcellin *et al*[10] (7.2 kPa for ⩾ F2, 8.1 kPa ⩾ F3, and 11.0 kPa for F4) as the reference values between 2 external studies that investigated the performance of TE and then set up the optimal cutoff LS values for each fibrosis stage in patients with CHB[10,11]. We identified 28 (18.0%) patients showing significant discordance between TE and LB. However, the IQR/M was not significantly different between patients with discordance and those with non-discordance (0.125 ± 0.083 *vs* 0.129 ± 0.079, $P = 0.831$), and the success rate also failed to show a significant difference.

Interestingly, the mean IQR/M of our study population was greatly reduced compared to that of subjects in Lucidarme *et al*[9] (0.30 in patients with discordance and 0.22 in those with non-discordance) and the proportion of patients with an IQR/M > 0.03 ($n = 7$, 4.5%) was very small. The reasons are not clear why the IQR/M in our study was reduced in comparison with that in the previous study, and was not selected as a discriminant factor to predict discordance. However, we think that the optimal cutoff value of the IQR/M will be lower than that from patients with CHC even if the IQR/M can be validated in the future for the Asian population with CHB, or that the IQR/M is not a predictor of discordance in the Asian population with CHB due to other potential confounders that overwhelm the influence of the IQR/M, such as the inhomogeneous histological features of CHB which often

Table 1 Normal liver stiffness values of transient elastography

	Asian study		European study				
	Kim *et al*[14]	Fung *et al*[19]	Corpechot *et al*[15]	Roulot *et al*[13]	Sirli *et al*[16]	Colombo *et al*[17]	Colombo *et al*[18]
Type	Full article	Full article	Letter	Full article	Full article	Abstract	Abstract
No. of subjects	69	28	71	429	144	327	746
Population	Liver and kidney donors	Liver donors	Healthy volunteers	Medical check-up	Healthy volunteers	Blood donors	Blood donors
Liver stiffness (kPa)	4.6 (mean)	4.6 (median)	4.8 (median)	5.5 (mean)	4.8 (mean)	4.9 (mean)	4.4 (median)
95th percentile (kPa)	4.7	-	-	8.6	-	7.8	6.7
BMI (kg/m^2)	22.6 (mean)	-	22.5 (median)	25.8 (mean)	-	-	-
Effects on TE							
Age	No	-	No	No	No	No	No
Gender	M = F	-	M > F	M > F	M > F	M = F	M > F
High BMI	No	-	No	Increased TE values	No	Increased TE values	No
Metabolic syndrome	-	-	-	Increased TE values	-	-	-
Fatty liver	-	-	-	-	-	Increased TE values	Increased TE values

BMI: Body mass index; TE: Transient elastography.

makes liver texture inherently macronodular. This would result in LS values that vary depending on the area of the liver and necroinflammatory activity, which has been demonstrated to raise LS values. Therefore, the validation of the IQR/M in the Asian population with CHB requires further studies using increased sample sizes to allow sufficient cases with a spectrum of IQR/M values.

Several issues still remain to be resolved regarding the Asian population with CHB: How many valid measurements do we need to maintain the performance of TE[12]? What is the optimal success rate of the TE measurement to accurately exclude unreliable LS values? Can the IQR/M alone be applied to increase the performance of TE without a sufficient number of valid measurements and an adequate success rate?

WHAT ARE THE NORMAL LS VALUES FOR THE ASIAN POPULATION

Before discussing data on TE in the Asian population with CHB, it is important to first confirm that TE can reliably identify patients with CLD from the normal population. If TE cannot, the clinical meaning of further analysis on the performance of TE in grading the severity of CLD would be significantly lessened. Despite the importance of this, to date, most studies on TE have focused on patients with CLD.

The concept of "what are normal LS values assessed by TE?" is a critical issue that should be addressed to determine whether TE can be used for screening the general population and for subsequent selection of high-risk patients who require periodic follow-up and adequate treatment measures. Indeed, concerns regarding normal LS values are increasing, and several studies (including 2 Asian studies) have proposed normal LS values despite variations in study design[13-19].

In a study by Corpechot *et al*[15], the authors found that the normal LS values in the healthy population were significantly higher in men than in women and that the median LS value was 4.8 kPa (range, 2.5-6.9 kPa). Roulot *et al*[13] examined a large cohort of 429 apparently healthy subjects to establish normal LS values [5.81 ± 1.54 kPa (range, 3.8-8.0 kPa) in men *vs* 5.23 ± 1.59 kPa (range, 3.3-7.8 kPa) in women, $P < 0.01$]. Another study conducted in Romania demonstrated that the mean values of TE in 144 normal subjects were 4.8 ± 1.3 kPa (range, 2.3-8.8 kPa) and that the normal LS values were significantly different between genders[16]. Colombo *et al*[17] also assessed the LS values of 327 voluntary blood donors and found that the mean LS value was 4.9 kPa (95% CI: 4.6-5.1 kPa) and the normal LS values showed no significant differences between genders. However, Colombo *et al*[18] in their follow-up study that enrolled more than 1000 healthy blood donors reported that the normal LS value was 4.4 kPa (95th percentile, 6.7 kPa) and that the male gender presents with increased LS values.

All the aforementioned studies were conducted in Europe. However, normal LS values in the Asian population are now available. Fung *et al*[19] reported a mean LS value in 28 healthy living-related liver donors of 4.6 kPa (range, 2.0-7.1 kPa), and all subjects had LS values of < 7.2 kPa, which indicated that they had no significant fibrosis. We have previously reported that the normal range of LS values was 3.9-5.3 kPa, which was calculated from 69 strictly selected living liver and kidney donors[14].

Table 1 indicates the results of all studies on normal LS values. The upper normal range of LS values was consistently lower than the range generally used for identifying significant liver disease (7-8 kPa) in the majority of previous studies[20-22]. These data demonstrated that TE can perform reliably in identifying high-risk subpopulations without an overlap between the normal and abnormal ranges of LS values. Interestingly, the mean LS values in Asian studies appear lower in comparison with European studies, and the effects of gender on the performance of TE are variable among studies. The discrepancy cannot be fully explained, because the complete histological data of the normal subjects were largely unavailable except a small

Table 2 Failure rate of transient elastography measurement

	Asian study				European study		
	Kim *et al*[14]	Chan *et al*[24]	Masuzaki *et al*[25]	Wong *et al*[26]	Ziol *et al*[2]	Marcellin *et al*[10]	Maimone *et al*[27]
Etiology	HBV	HBV	HCV	HBV	HCV	HBV	HBV
Total patients (*n*)	74	1136	876	487	327	202	230
Excluded patients (*n*)	5	30	10	34	76	29	10
Excluded patients due to TE failure (*n*)	1	30	10	17	23	14	10
TE failure rate (%)	1.4	2.6	1.1	3.5	7	6.9	4.3
Enrolled patients (*n*)	69	1106	866	453	251	173	220
Age (yr)	38.9 (mean)	47 (mean)	66.2 (mean)	37 (median)	47.5 (mean)	40.1 (mean)	45.2 (mean)
Male, *n* (%)	35 (50.7)	-	398 (46.0)	270 (60.0)	155 (61.8)	115 (66.5)	99 (45.0)
BMI (kg/m^2)	22.6 (mean)	-	22.5 (mean)	22.4 (median)	23.9 (mean)	24.5 (mean)	-
Failure criteria							
Success rate	< 60%	< 60%	< 60%	< 60%	< 60%	< 50%	< 60%
The number of VMs	< 10 VMs	< 10 VMs	< 8 VMs	< 10 VMs	< 10 VMs	< 7 VMs	< 10 VMs

HBV: Hepatitis B virus; HCV: Hepatitis C virus; TE: Transient elastography; BMI: Body mass index; VM: Valid measurement.

number of liver donors in the studies by our group[14] and Fung *et al*[19]. Lower mean BMI, younger age, and lower prevalence of metabolic syndrome in the Asian studies can explain, at least in part, the lower mean LS values observed. Another reasons are that other confounders of TE performance, such as high alanine aminotransferase (ALT) levels and fatty liver, were not sufficiently excluded[17,18] and that the hepatic imaging studies and/or cardiologic evaluation were not fully completed in European studies[13,16]. These reasons may have increased the normal range of LS values in the European studies.

More carefully designed studies with a large number of subjects are thus still required to fully assess the normal LS values for both Western and Asian populations. However, it should be remembered that the actual normal range of LS values cannot be precisely defined without a definition of a "normal liver", sufficient histological evaluation of normal subjects, and further stratified analysis with lifestyle factors between genders (such as alcohol consumption). If these issues are resolved, we can identify the true normal LS value and use this as a reference for future studies.

INFLUENCE OF BMI ON THE PERFORMANCE OF TE IN THE ASIAN POPULATION

Previous work has demonstrated that BMI can affect the performance of TE. This can be explained in 2 ways. First, BMI influences the failure rate of TE, and second, BMI acts to increase LS values. Although previous studies have reported that the success rate of TE decreases in subjects displaying higher BMI[13,23], TE failure may not be significant, because these patients were largely excluded from the analysis. However, in another point of view, this effect of high BMI on TE failure can be a significant pitfall of TE, as the majority of patients with high BMI have an increased chance of suffering from fatty liver and, if TE examination fails, they miss the opportunity to receive rapid and non-invasive methods to exclude significant fibrosis. From the knowledge of the varying failure rates between Asian and European study populations, we can discern the possible influence of BMI on TE failure rate according to the study population.

Table 2 summarizes the failure rates of previous studies conducted in Asian and European populations. Overall, TE failure rates and BMI both appear to be lower in Asian compared to European studies, indicating that the lower BMI found in Asian populations is possibly associated with a lower TE failure rate.

In spite of debate, several European studies have reported that a high BMI (which might cause hepatic steatosis) could potentially increase LS values[13,17], although research to date has not determined whether steatosis itself increases LS value in patients with chronic viral hepatitis[28-30]. For the Asian population, the effects of high BMI on TE performance have not been fully validated, because most Asian studies on TE lack sufficient numbers of patients with high BMI (> 30 kg/m^2). In our previous study, BMI did not influence LS values, although our study population was younger and showed lower BMI values than European studies[14]. This potential effect of high BMI on TE performance should therefore be further investigated for the Asian population.

INFLUENCE OF METABOLIC SYNDROME ON THE PERFORMANCE OF TE IN THE ASIAN POPULATION

Recently, the effects of metabolic syndrome (MS) on TE have been examined in a French study[13]. Here, BMI > 30 kg/m^2 was more frequent among subjects with MS than among normal subjects (49.1% *vs* 8.9%, respectively, $P < 0.001$). The mean LS value was higher in subjects with MS compared to controls (6.51 ± 1.64 kPa *vs* 5.33 ± 1.51 kPa, $P < 0.001$). In a multivariate analysis, LS values were still significantly different between subjects with and without MS, irrespective of BMI and other variables. Because MS

has been demonstrated to increase LS values significantly, and hepatic steatosis which can result in histological progression to cirrhosis and hepatocellular carcinoma is strongly associated with MS[31-33], a potential role of TE in detecting MS was proposed. However, 88% of the subjects with MS had LS values within the defined normal range. This result indicated that not only could TE not precisely perform in the diagnosis of MS, but also that hepatic steatosis differentially affected by MS may have influenced LS values differently for different individuals. One drawback of the study[13] was a lack of ultrasonographic evaluation of hepatic steatosis. It is thus unclear whether the increased LS values in the subjects with MS were dependent on MS itself or on hepatic steatosis associated with MS. Other studies in Italy[17,18] attempted to overcome this drawback by including ultrasonographic evaluation of the healthy blood donors and concluded that the severity of hepatic steatosis was independently related to LS values.

The association between MS or hepatic steatosis and LS values was investigated primarily in healthy subjects, because the effects of MS or hepatic steatosis on TE can be attenuated if the study population presented with background fibrotic liver (the most significant factor for high LS values). Therefore, it is difficult to identify the main factors that increase LS values among MS, hepatic steatosis, associated steatofibrosis, and background fibrosis, if the study enrolls patients already presenting with CLD.

As mentioned above, only one published European study reporting the effects of MS on TE in healthy individuals is currently available. Furthermore, no available European and Asian data address the effects of MS on TE performance in patients with chronic viral hepatitis. Considering the different clinicopathological course of hepatic steatosis in patients with CHC and CHB and the varied distribution of body fat according to race, further studies on the effects of MS and hepatic steatosis should be performed with the Asian populations.

INFLUENCE OF ALT ON THE PERFORMANCE OF TE IN THE ASIAN POPULATION WITH CHB

The most important confounding factor of TE is serum ALT levels. Hepatic inflammation, as reflected by higher ALT levels, tends to increase LS values[11,20,34]. Even minor changes in ALT levels have been shown to influence LS values[35]. Because HBV, unlike HCV, frequently exhibits acute ALT flares[36,37], the interpretation of LS values becomes increasingly difficult in the setting of CHB when major fluctuations of necrosis and inflammatory activity occur[24,38]. It thus becomes more relevant to consider ALT levels when examining TE in the Asian population with CHB.

Considering the significant effects of high ALT levels, several Asian studies with CHB patients attempted to establish varying cutoff LS values according to ALT levels. Chan *et al*[11] proposed ALT-based algorithms when interpreting LS results, and established different optimal cutoff values according to ALT levels (one group with normal ALT *vs* the other group with ALT > upper limit of normal (ULN) and ≤ 5 × ULN). In our previous study, we also stratified the study population according to ALT levels and calculated the optimal cutoff values for each group[34]. For patients with normal ALT levels, 6.0, 7.5 and 10.1 kPa were selected as the optimal cutoff values for ≥ F2, ≥ F3, and F4, respectively, whereas 8.9, 11.0, and 15.5 kPa were selected in those with ALT > ULN and ≤ 2 × ULN. Considering this unreliability of TE performance in patients with high ALT levels, more recent studies began to exclude patients displaying ALT > 5 × ULN for analysis[24]. In addition, the optimal time interval for TE to recover its reliability in patients who experience acute exacerbation of CHB was recently reported[38].

Although it seems reasonable to use different cutoff values according to ALT levels, large-scale validation of these LS values has not been performed for Asian patients with CHB. Furthermore, some issues still remain unresolved, such as how we stratify patients with CHB according to ALT levels to establish the cutoff values, and who should be excluded for TE examination because of unreliability of TE among patients with high ALT levels.

DIAGNOSTIC PERFORMANCE OF TE IN THE ASIAN POPULATION WITH CHB

Few studies have investigated the performance of TE in the Asian population with CHB[4,11,39-43]. The characteristics of studies and the identified performance of TE to predict significant fibrosis and cirrhosis are summarized in Tables 3 and 4 (Marcellin *et al*[10] is listed for comparison with other Asian studies). The listed Asian studies were selected if they evaluated TE in Asian populations with CHB, they used LB as a reference standard, and they assessed the diagnostic accuracy of TE [using area under the receiver operating characteristic curve (AUROC)] for F ≥ 2 or F = 4 fibrosis stage and/or diagnostic indexes (sensitivity, specificity, positive predictive value, or negative predictive value based on some cutoff LS values). Most Asian studies were conducted in Korea and China (Hong Kong) and some studies were available in abstract form from Korea, Singapore, and Thailand.

The AUROCs for predicting F ≥ 2 fibrosis and F = 4 fibrosis stage in patients with CHC were reported as 0.79-0.83 and 0.97-0.95, respectively in the studies by Ziol *et al*[2] and Castera *et al*[44]. However, the AUROCs in Asian studies seems to be lower than those reported in European studies (0.76-0.88 for F ≥ 2 and 0.80-0.93 for F4, Table 4), although TE diagnosed cirrhosis more accurately than significant fibrosis in Asian studies.

Overall, TE is accepted as a promising and accurate tool for the early detection of cirrhosis irrespective of the etiology of CLD, although the optimal cutoff values remain debatable. The range of the optimal cutoff values for diagnosing HBV-related cirrhosis in the Asian population were between 9.0 and 10.1 kPa based on the full-length articles[4,11,39] (Table 4) and the range for cirrhosis was less than 11.0 kPa, which is consistently lower than in

Table 3 Characteristics of studies evaluating the performance of transient elastography for the diagnosis of liver fibrosis in patients with chronic hepatitis B

	Type	Country	Total sample (*n*)	Sample size (*n*)	Excluded due to failure		Age (yr)	Male (%)	BMI (kg/m^2)	LB length (mm)	Staging
					TE (reason)	LB (reason)					
Kim *et al*[4]	Original	Korea	194	103	0 (SR < 60%, < 10 VMs)	4 (< 10 mm, < 10 PTs)	40.0	80.2	23.8	16.7	METAVIR
Chan *et al*[11]	Original	China	186	161	1 (SR < 60%, < 10 VMs)	22 (< 15 mm, < 6 PTs)	45.0	76.0	24.0	19.0	METAVIR
Kim *et al*[36]	Original	Korea	130	130	0 (SR < 60%, < 10 VMs)	0 (< 10 mm, < 6 PTs)	42.5	79.2	25.3	14.5	METAVIR
Marcellin *et al*[10]	Original	France	202	173	14 (SR < 50%, < 7 VMs)	15 (< 10 PTs)	40.1	66.5	24.5	16.6	METAVIR
Chang *et al*[40]	Abstract	Singapore	35	33	2 (obesity, narrow ICS)	0 (-)	43.0	-	25.6	-	Ishak
Tanwandee *et al*[41]	Abstract	Thailand	104	104	0 (-)	0 (-)	44.0	63.0	23.6	-	METAVIR
Choi *et al*[42]	Abstract	Korea	48	48	0 (-)	0 (-)	41.7	58.3	23.3	-	-
Chang *et al*[43]	Abstract	Singapore	88	84	3 (-)	1 (-)	49.0	71.6	-	-	-

TE: Transient elastography; VM: Valid measurement; LB: Liver biopsy; BMI: Body mass index; SR: Success rate; PT: Portal tract; ICS: Intercostal space.

Table 4 Histologic distribution and the performance of transient elastography for the diagnosis of liver fibrosis in patients with chronic hepatitis B

	n (%)					METAVIR and other scoring system (≥ F2/F4)							
	F0	F1	F2	F3	F4	AUROC	Cutoff (kPa)	Se (%)	Sp (%)	PPV (%)	NPV (%)	LR (+)	LR (-)
Kim *et al*[4]	0	9 (9.9)	33 (36.3)	10 (11.0)	39 (42.9)	-/0.803	-/9.7	-/82	-/62	-/63	-/76	-/4.97	-/0.13
Chan *et al*[11]	10 (6.2)	27 (16.8)	47 (29.2)	37 (23.0)	40 (24.8)	-/0.93	-/9	-/98	-/75	-/57	-/98	-/1	-/0.01
Kim *et al*[36]	0	10 (7.7)	37 (28.5)	16 (12.3)	67 (51.5)	-/0.84	-/10.1	-/76	-/81	-/76.1	-/80.9	-/-	-/-
Marcellin *et al*[10]	16 (9.2)	70 (40.5)	44 (25.4)	29 (16.8)	14 (8.1)	0.81/0.93	7.2/11	70/98	83/75	80/57	73/98	4/1	-/0.01
Chang *et al*[40]	7 (20.0)	16 (45.7)	F2-3 (5, 14.3)		7 (20.0)	-/-	11.8/-	90/-	78/-	-/-	-/-	-/-	-/-
Tanwandee *et al*[41]	-	-	-	-	-	0.757/-	6.9/7.3	70/93	79/61	82/31	66/98	-/-	-/-
Choi *et al*[42]	-	-	-	-	-	0.88/0.86	7.7/10.4	88/79	88/83	-/-	-/-	-/-	-/-
Chang *et al*[43]	-14.8	-30.7	-14.8	-21.6	-17.1	0.801/-	8.8/-	-/-	-/-	-/-	-/-	-/-	-/-

AUROC: Area under the receive operating characteristic curve; Se: Sensitivity, Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; LR: Likelihood ratio.

patients with CHC[45,46]. These findings can be explained in several ways. The histopathological characteristics of CHC (including portal lymphoid follicles, bile duct damage, lobular activity, and steatosis) may contribute to the variations in cutoff values compared with those in patients with CHB[47]. Another explanation is that the total fibrotic material in CHB may be lower than that in CHC, because CHB tends to make the liver macronodular and heterogeneous[48]. Other researchers have proposed that the different types and extent of liver inflammatory infiltrate within the liver may account for the different cutoff LS values between CHB and CHC[20].

The performance of TE in prediction of cirrhosis is acceptable for the Asian population with CHB, but increased performance in prediction of significant fibrosis and subsequent precise staging, particularly for patients with CHB who are candidates for antiviral treatment, is needed and should be pursued through future studies. This is particularly important, because the decision to start antiviral treatment and the type of antiviral agents to be used may be affected by fibrosis stage.

CONCLUSION

Because of limitations of LB, prohibiting its routine use for the evaluation of liver fibrosis in patients with CHB, interest in the use of noninvasive TE has increased. Ideally, TE can be used to screen the general population to detect high-risk patients with potential liver disease, to identify patients with significant fibrosis who could benefit from the initiation of antiviral treatment, to select patients with cirrhosis who are at a high risk of developing HCC[25], and to identify patients with cirrhosis and portal hypertension. To date, although numerous European studies have demonstrated that this is possible, data on TE for the Asian population are scarce.

We are now fully armed with nuggets of knowledge that the etiologies of CLD, BMI, MS, cardiac function, cholestasis, hepatic steatosis, and ALT levels can influence the performance of TE, and also that several confounders vary between Asian and European populations, for example, the etiology of CLD, major fluctuation in ALT levels, and the prevalence of high BMI and MS. However, because almost all information on TE has thus far originated from European data, for the Asian population with CHB, TE is not popular clinically. Therefore, further studies tailored to the Asian population with CHB will restore the confidence of Asian clinicians to utilize TE.

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REVIEW

Consequences of *Helicobacter pylori* infection in children

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Abstract

Although evidence is emerging that the prevalence of *Helicobacter pylori* (*H. pylori*) is declining in all age groups, the understanding of its disease spectrum continues to evolve. If untreated, *H. pylori* infection is lifelong. Although *H. pylori* typically colonizes the human stomach for many decades without adverse consequences, children infected with *H. pylori* can manifest gastrointestinal diseases. Controversy persists regarding testing (and treating) for *H. pylori* infection in children with recurrent abdominal pain, chronic idiopathic thrombocytopenia, and poor growth. There is evidence of the role of *H. pylori* in childhood iron deficiency anemia, but the results are not conclusive. The possibility of an inverse relationship between *H. pylori* and gastroesophageal reflux disease, as well as childhood asthma, remains a controversial question. A better understanding of the *H. pylori* disease spectrum in childhood should lead to clearer recommendations about testing for and treating *H. pylori* infection in children who are more likely to develop clinical sequelae.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is one of the most common chronic bacterial infections world-wide, and it is currently estimated that approximately half of the world's population is infected with the bacterium[1,2]. However, the prevalence of *H. pylori* is not homogeneous world-wide[1,3]. In western countries, the prevalence of infection has been decreasing during the past few decades[4-6]. *H. pylori* infection is acquired early in life (almost always before the age of 10 years), and in the absence of antibiotic therapy, it generally persists for life[1].

It is widely accepted that *H. pylori* infection is the main etiological factor for gastritis and peptic ulcer[3]. Its eradication is associated with healing of these diseases and significant reduction of ulcer recurrence and rebleeding[7,8]. Several studies have demonstrated that inflammation caused by *H. pylori* infection might contribute to the development of adenocarcinoma of the stomach; moreover, it has been involved in the development of low-grade B-cell lymphoma of gastric mucosa-associated lymphoid tissue

type (MALT)[3,9]. Recently, a potential role of *H. pylori* infection in other digestive diseases (gastroesophageal reflux disease; GERD) as well as several extra-intestinal pathologies [iron deficiency anemia (IDA), growth retardation, idiopathic thrombocytopenic purpura (ITP), asthma and allergic disorders] has been suggested[10]. The postulated role of *H. pylori* in the pathogenesis of extra-intestinal manifestations is based on the facts that: (1) local inflammation has systemic effects; (2) *H. pylori* gastric infection is a chronic process that lasts for several decades; and (3) persistent infection induces a chronic inflammatory and immune response that is able to induce lesions both locally and remote to the primary site of infection[11].

The aim of this report is to provide a critical review of the available literature about digestive and extradigestive manifestations of *H. pylori* infection in children. Pertinent articles have been identified through a MEDLINE search. Studies published in English during the past two decades have been identified and reviewed.

GASTRITIS AND PEPTIC ULCERS

During childhood, *H. pylori* is associated with predominant antral gastritis, and duodenal ulcers[12-14]. Successful eradication of *H. pylori* markedly reduces the rate of recurrence of duodenal ulcers in affected children[2,15,16]. Gastric ulcers are much less common in children than they are in adults[17].

A pooled analysis of early reports (1983-1994) has demonstrated that the rate ratio of antral gastritis for children with *H. pylori* infection (compared with uninfected subjects) ranged from 1.9 to 71.0 (median, 4.6)[17]. The prevalence of *H. pylori* in children with duodenal ulcer was high (range, 33%-100%; median, 92%) compared with children with gastric ulcer (range, 11%-75%; median, 25%)[17]. Thus, there was strong evidence for an association between *H. pylori* infection and antral gastritis and duodenal ulcer in children; there was weak evidence for an association with gastric ulcer. Nevertheless, a subsequent retrospective study (1995-2001) from Japan has confirmed that the prevalence of *H. pylori* was very high in antral (nodular) gastritis and duodenal ulcer (98.5% and 83%, respectively), but it has also demonstrated that *H. pylori* was a definite risk factor for the development of gastric ulcer, although the prevalence of infection did not reach 50%[18]. *H. pylori* was significantly linked to duodenal and gastric ulcers in the age group 10-16 years, but not ⩽ 9 years.

More recently, a decreasing proportion of *H. pylori*-positive peptic ulcers in adults has been observed, along with a decrease in the prevalence rate of the infection[19]. In children, there have been few data published in the literature to investigate the trend of *H. pylori* prevalence in peptic ulcer[20-23]. In a prospective European multicenter pilot study on the incidence of gastric and duodenal ulcer disease in children, Kalach *et al*[20] have found that ulcers occurred in 10.6% of cases, with *H. pylori* infection in only 26.7% of these. From January 2001 to December 2002, information on 518 children was collected from the pediatric European register for treatment of *H. pylori*[21]. At endoscopy, 454 children had *H. pylori*-associated gastritis and 64 had an ulcer (12.3%). However, this series included children from Russia and they had a significantly higher prevalence of peptic ulcer (35% *vs* 6.7% in the remainder of European children, $P < 0.0001$; OR: 7.5; 95% CI: 4-13). Thus the prevalence of *H. pylori*-positive ulcers in children differed between countries, and this was not completely explained by the prevalence of the infection in the population studied[22]. In a retrospective review (1998-2006) of 619 Chinese children who had undergone upper endoscopy for investigation of upper gastrointestinal symptoms, Tam *et al*[23] have found that 43 (6.9%) had peptic ulcer. Of these 43 patients, 37 and six had duodenal and gastric ulcer, respectively. The prevalence of *H. pylori* infection was 56.8% (21/37) in duodenal ulcer and 33.3% (2/6) in gastric ulcer. When they arbitrarily divided the study period into two, 1998-2001 and > 2002, no significant difference in the prevalence of *H. pylori* infection between the two periods was found.

GASTRIC MALIGNANCIES

In relation to *H. pylori*-associated gastric malignancies in children, there have been a few cases of gastric MALT lymphoma[14,24,25], but there have been no reports of adenocarcinoma.

There is evidence to support an association between long-standing *H. pylori* infection, gastric atrophy, and intestinal metaplasia with the development of intestinal-type and undifferentiated adenocarcinomas in adults[3]. It has been suggested that chronic gastritis, gastric atrophy, intestinal metaplasia and gastric cancer develop progressively, stepwise over decades, in predisposed individuals infected by *H. pylori*[26]. However, gastric atrophy and intestinal metaplasia have indeed been described in children living in countries with high gastric cancer incidence[27], and they are sometimes found in very young subjects[28-30]. These findings provide support to the hypothesis that host genetic factors that affect the inflammatory and immune response to *H. pylori* infection might determine why some individuals infected with this bacterium develop precancerous lesions and gastric carcinoma while others do not[31,32]. Thus, it is probable that the prevalence of gastric atrophy and intestinal metaplasia varies according to the geographic/genetic origins as well as environmental factors[27-30,33-40], as shown in Table 1. Yet, sampling problems exist. In fact, the non-systematic search during pediatric gastroscopy for these histological states might mask their true prevalence[32]. Most reported studies of the histological features of *H. pylori* infection in children have used either random biopsies[41] or a small number of targeted biopsies[29,34,42,43], taken primarily from the antrum[29,44]. In most studies, the identification of atrophy has been focused on the presence of intestinal metaplasia or has been ill defined[29].

In clinical practice, the updated Sydney system is widely used for grading gastric histopathological findings (density

Table 1 Prevalence of gastric atrophy in children

Author (yr)/country	Updated Sydney System	Mean age (range, yr)	No. of patients		Gastric atrophy and/or intestinal metaplasia (%)	
			H. pylori+	*H. pylori*-	*H. pylori*+	*H. pylori*-
Whitney *et al*[28] (2000)/USA	Yes	10.7 (1-21)	42	0	Antral: 16.6/0 Fundic: 2.3/0	-/-
Kolho *et al*[33] (2000)/Finland	Not reported	9.5 (2-16)	71	0	0/0	-/-
Campbell *et al*[34] (2001)/Gambia	Yes	1.4 (-/-)	21	16	0/0	0/0
Guiraldes *et al*[35] (2002)/Chile	No	12.2 (5-17)	59	14	0/0	0/0
Oztürk *et al*[36] (2003)/Turkey	Yes	12.2 (6-16)	18	9	Antral: 72.2/77.7	11.1/0
Guarner *et al*[29] (2003)/USA	Yes	- (1-17)	19	45	Antral: 52.6/15.7 Fundic: 0/5.2	22.2/0 0/0
Usta *et al*[37] (2004)/Turkey	Yes	11.8 (4-17)	175	0	2.2/1.1	-/-
Levine *et al*[38] (2004)/Israel	Not reported	14.2 (-/-)	55	40	0/0	2.5/0
Ricuarte *et al*[27] (2005)/Colombia, Korea	No	12.0 (4-18) 14.0 (8-18)	97 10	18 48	16.4[1] 0	[1] 0
Kato *et al*[39] (2006)/Japan	Yes	11.3 (1-16)	131	65	Antral: 51.9/4.6 Fundic: 34.8/0	10.8/4.6 8.3/4.2
Tutar *et al*[30] (2009)/Turkey	Yes	1.3 (0.1-2.0)	40	112	2.5/0	0/0
Kalach *et al*[40] (2009)/France	Yes	5.3 (0.1-17.7)	66	553	0/0	0.2/0.2

[1]Atrophic mucosa: 31% as intestinal metaplasia; 63% as pseudopyloric metaplasia; 6% as both. *H. pylori*: *Helicobacter pylori*.

of *H. pylori* organisms, acute and chronic inflammation, atrophy and intestinal metaplasia)[45]. Although this system has been validated in adult patients, interobserver variability is still a problem, primarily in the evaluation of mucosal atrophy[46,47]. Thus, further validation of atrophy parameters needs to be obtained for pediatric biopsy samples[29]. In contrast, metaplastic epithelium is easily detected by the pathologist, owing to the characteristic goblet cells[32].

In adults, five gastric biopsy samples are recommended (two antral, two corporeal and one from the angulus)[45], but no consensus is available about the optimal number and site location of gastric biopsies in children. Clinical practices in this domain are very heterogeneous, which could in part account for the different prevalence figures of atrophic gastritis in children[32]. Of note, in a study of 173 children from countries with high gastric cancer incidence, Ricuarte *et al*[27] emphasized the importance of biopsy site location for identifying the presence of corpus atrophy. In children, atrophy is only identified in biopsies taken near the normal antrum-corpus junction, which is consistent with the notion that atrophy progresses as an advancing antrum-corpus border[27]. Therefore, identification and characterization of the natural history of *H. pylori* gastritis requires, in addition to biopsies that target the antrum and the cardia, targeted biopsies to include the lesser and greater curvature of the corpus, starting just proximal to the anatomical junction of the antrum and corpus. Unfortunately, the sites recommended by the updated Sydney system can identify corpus atrophy only when it is extensive.

Studies from the 1990s have established that the development of low-grade gastric MALT lymphoma is strongly associated with chronic *H. pylori* gastritis. In two large series of adult patients, *H. pylori* was detected by histological examination in 92% and 100% of those with gastric MALT lymphoma[48,49]. Recognition of the responsiveness of MALT lymphoma to antibiotic therapy aimed at eradication of *H. pylori* has changed the approach to its management. Remission rates in the literature range from 60% to 80%, although recurrence can be expected in 5% of cases[50-53]. MALT lymphoma occurs commonly in middle and old age; only a small number of cases have been reported in both immunocompetent and immunocompromised children[14,24,25]. Individual case reports have described the regression of MALT lymphoma after *H. pylori* eradication therapy alone[24,25].

FUNCTIONAL DYSPEPSIA

The role of *H. pylori* infection as a cause of non-ulcer or functional dyspepsia has been one of the most debated controversies in the medical community since the discovery of this bacterium.

Dyspepsia is very common in children with chronic or recurrent abdominal pain (RAP), with as many as 80% reporting this symptom[54]. The relation between RAP in childhood and *H. pylori* infection is not clear[17]. Pediatric studies are limited by the lack of a clear definition for RAP or by the use of nonspecific criteria for the diagnosis of chronic abdominal pain[54]. A pooled analysis of early reports (1983-1994) has demonstrated that prevalence rates of infection in children with RAP were inconsistent (range, 0%-81%; median, 22%),with lower rates (range, 0%-9%; median, 6%), in children who met Apley's criteria (i.e. at least three discrete episodes of abdominal pain of sufficient severity to interrupt normal daily activities or performance occurring over a period of $\geq$ 3 mo)[17].

In adults, several controlled trials have shown a vague connection between *H. pylori* colonization and dyspeptic symptoms[55]. In children, controlled randomized treatment studies have been scant. The results of two uncontrolled trials have suggested improvement of clinical symptoms after treatment of *H. pylori* infection[56,57]. However, the double-blind, randomized placebo-controlled trial by Ashorn *et al*[58] has suggested that RAP is not an indication

for a test and treatment strategy for *H. pylori* infection in children. In fact, in that study, at 52 wk, dyspeptic symptoms improved to the same extent in the treatment group and in children who received placebo, irrespective of the healing of gastritis, which was more commonly achieved along with eradication[58]. Nonetheless, due to the limited number of patients who could finally be included, the results by Ashorn *et al*[58] have to be interpreted with care. Large-scale multicenter trials performed in children are still needed to answer definitively the question whether a connection exists between *H. pylori* infection and RAP. Neither did the results of a very recent study give support for the use of *H. pylori* eradication in children with RAP[59]. Based on a meta-analysis of 38 studies between 1966 and 2009, the study by Spee *et al*[59] has found no association between RAP (fulfilling Apley's criteria) and *H. pylori* infection in children. However, the authors have demonstrated that children who are referred to a gastroenterologist with unspecified abdominal pain (i.e. including children who do not fulfill Apley's criteria) or pain in the epigastric region are at 2-3-fold higher risk for *H. pylori* infection than children without these symptoms. Thus, these authors have postulated that unspecified abdominal pain in a hospital-based setting and epigastric pain in general might be associated with (acute) *H. pylori* infection. The potential for *H. pylori* to cause clinical symptoms that arise from gastric infection, in the absence of mucosal ulceration, requires additional studies using a strict study group definition. Functional dyspepsia and non-ulcer dyspepsia (i.e. epigastric pain in the absence of mucosal ulceration at esophago-gastro-duodenoscopy) must be evaluated as separate entities.

GASTROESOPHAGEAL REFLUX DISEASE

The interaction between *H. pylori* infection and GERD has been widely debated in the literature over the past decade, and the hypothesis that eradication of *H. pylori* leads to increased GERD has been the subject of many publications with contradictory conclusions in children as well as in adults[38,60-76]. There are limited data in children because the prevalence of *H. pylori* infection is low and no randomized controlled trials have been conducted; therefore, inferences about the effects of *H. pylori* eradication and GERD need to be drawn from studies on adult patients[77].

H. pylori has been found to be inversely correlated with the prevalence of GERD, and certain studies have shown aggravation of esophagitis after eradication[60-66]. Suggested mechanisms include presence of atrophic or significant body gastritis that leads to a post-eradication increase in acid output; decreased buffering as a result of elimination of *H. pylori*, which produces ammonia *via* bacterial urease; masking of reflux by acid neutralizing medications given for *H. pylori*-related disease; and increased appetite with weight-gain- mediated reflux. These observations are controversial, because several studies have not found an association between eradication of *H. pylori* and reflux disease[38, 66,70,74,78-80].

Most studies aimed at evaluating the effect of *H. pylori* eradication on reflux in adults have used selected populations such as those with duodenal ulcer or patients with GERD before eradication[63,65,78,80]. The spectrum of risk factors found in adults, such as atrophic gastritis, duodenal ulcer, or significant esophagitis, might influence the outcome of the study. These factors are less common in children, therefore, these results might not be relevant to the decision to eradicate *H. pylori* when found in children[38].

Dent has proposed that the effect of *H. pylori* eradication on GERD is most likely determined by the population studied[67]. Acid secretion in predominant antral gastritis with preserved body mucosa is hyper-responsive, thus enabling increased duodenal or esophageal injury. In these patients, eradication should improve or not affect GERD. This hypothesis is consistent with the results of other studies that have shown improvement in GERD symptoms in patients with duodenal ulcer[80,81]. However, in patients with atrophic gastritis or severe body gastritis, *H. pylori* eradication might result in increased acid secretion. Children and adolescents are more likely to behave like the first group, with predominant antral gastritis[38].

The risks and benefits of *H. pylori* eradication are less well-defined for patients with gastritis alone, and vary according to the severity and pattern of gastritis[67]. Although this is the patient group most likely to develop reflux esophagitis[63], the risks from this are outweighed by those of continued *H. pylori* infection. Reflux esophagitis following *H. pylori* eradication is believed to carry little risk and, in particular, not to lead to intestinal metaplasia. By contrast, the risks of continued *H. pylori* infection are relatively high in patients who have had an episode of chronic duodenal, gastric or gastroduodenal ulceration[67]. What is certain, is that *H. pylori* is a major risk factor for non-cardia gastric adenocarcinoma[82], and children with this infection have at least a fivefold increased risk of developing stomach neoplasia in later life. This risk is likely to be reversed with *H. pylori* eradication[77]. In a study of high-risk adults, no reduction in gastric cancer risk at the end of 7.5 years of follow-up was observed in *H. pylori* carriers who had previously undergone eradication therapy[83]. However, in a subgroup analysis of patients who had no precancerous lesions at baseline, cancer risk was significantly reduced[83]. Additional studies have suggested that prevention of gastric cancer might be possible in infected individuals without precancerous lesions[84,85]. Therefore, children could well be the group to target in an effort to prevent the future development of gastric cancer.

IRON DEFICIENCY ANEMIA

In addition to the already known causes of IDA, over the past two decades, an association between *H. pylori* and pediatric IDA has been established[86-92]. However, the issues of whether *H. pylori* infection is linked causally to IDA in children and whether treatment or resolution of *H. pylori* infection would improve iron stores or resolve IDA in children are still matters of great debate. In 1991,

Table 2 Randomized trials of *Helicobacter pylori* eradication for iron deficiency anemia and iron deficiency in children

Author (yr)/country	No. of children with IDA/*H. pylori*	Follow-up (No. of children)	Outcome	
			IDA	ID
Choe *et al*[89] (1999)/Korea	43/25	8 wk (18)	Hb increased with: eradication + iron, eradication + placebo *vs* iron + placebo ($P = 0.0086$)	No significant differences in serum iron or ferritin
Sarker *et al*[106] (2008)/Bangladesh	260/200	3 mo (260)	IDA persisted with: eradication + iron, 11%; eradication alone, 33%; iron alone, 0%; placebo, 45%	ID persisted with: eradication + iron, 19%; eradication alone, 65%; iron alone, 7%; placebo, 78%
Gessner *et al*[105] (2006)/Alaska	219/219	14 mo (201)	IDA persisted with: eradication + iron, 22%; iron alone, 14%	ID persisted with: eradication + iron, 65%; iron alone, 72%
Fagan *et al*[104] (2009)/Alaska	219/219	40 mo (176)	IDA persisted with: eradication + iron, 5%; iron alone, 19%	ID persisted with: eradication + iron, 52%; iron alone, 58%

Hb: Hemoglobin; ID: Iron deficiency; IDA: Iron deficiency anemia; *H. pylori*: *Helicobacter pylori*.

an association between *H. pylori* infection and IDA due to microscopic blood loss was described in a 15-year-old girl with *H. pylori*-positive chronic active hemorrhagic gastritis, who showed no signs of gastrointestinal symptoms[88]. Two years later, Dufour *et al*[93] reported a 7-year-old child who presented with *H. pylori*-associated chronic antral gastritis without evidence of hemorrhage or clinical symptoms other than sideropenic anemia, which was refractory to oral iron administration and subsided after *H. pylori* eradication. These case reports were followed by other studies that have identified an association between *H. pylori* infection and pediatric unexplained or refractory IDA, and have indicated improvement of iron stores and anemia after successful *H. pylori* eradication[94-100]. Yet, some pediatric studies have implicated *H. pylori* as a cause of IDA that is refractory to oral iron treatment[94,95,99,100]. Thus, the above studies have supported a clinically significant influence of *H. pylori* infection on body iron stores and have led to a recommendation for *H. pylori* eradication in infected individuals with unexplained IDA[101-103]. However, small sample sizes, lack of control groups, and other methodological issues, including the use of validated measures of active *H. pylori* infection such as biopsy-related tests to confirm *H. pylori* infection, are among factors that have limited the interpretation and ability to generalize the importance of the results of these studies in children.

To the best of our knowledge, only four population-based randomized trials of the effect of *H. pylori* infection treatment on IDA have been performed in children[89,104-106], as shown in Table 2. Three of them lacked true placebo groups[89,104,105]. Choe *et al*[89] have demonstrated a beneficial effect of *H. pylori* eradication therapy plus iron or placebo in increasing hemoglobin levels. However, no significant differences among study groups were found at 8 wk follow-up for serum iron level, total iron-binding capacity, and ferritin level. Sarker *et al*[106] have shown, in a relatively large cohort of Bangladeshi children with IDA, a similar improvement of IDA as well as iron deficiency, with anti-*H. pylori* therapy plus iron compared with iron therapy alone. Therefore, the improvement of iron status in children who receive combined therapy could be attributed to the effect of iron rather than anti-*H. pylori* therapy. The findings from the Bangladeshi children corroborated well those of the study from rural Alaska, which showed that treatment and resolution of *H. pylori* infection did not substantially decrease levels of iron deficiency or mild anemia at 14 mo after treatment initiation, despite the relatively low rate of reinfection once the initial infection resolved[105]. When an additional follow-up evaluation at 40 mo after treatment initiation was performed, it was found that sustained resolution of *H. pylori* infection substantially reduced the prevalence of mild IDA but modestly improved iron status[104].

How can *H. pylori* gastritis cause IDA? Several theoretical mechanisms have been proposed to explain the possible relationship between *H. pylori* infection and decreased iron stores. It appears that chronic gastrointestinal blood loss is not the likely culprit, because most published cases and case series have found no bleeding lesions at the time of endoscopy, and have reported negative testing for fecal occult blood[107]. Another explanation for a relationship between *H. pylori* infection and IDA involves the possible effect of *H. pylori* gastritis on gastric acid secretion and iron absorption. Non-heme iron accounts for 80% of dietary iron in industrialized countries[107]. Crucial to the effective absorption of non-heme iron is hydrochloric acid in acid secretions. Reduction of the ferric to ferrous form is dependent upon the pH of the gastric juice, and reduction to the ferrous form facilitates membrane transport[108]. An important promoter of iron absorption is ascorbic acid, which appears to act in two ways: by promoting reduction to the ferrous form, and by forming an absorbable molecular complex with ferric iron, which is insoluble at pH > 5[107,109]. Gastric acid hyposecretion results from atrophy of the gastric glands and fundic mucosa, which has been associated with chronic *H. pylori* infection[107]. It has been shown that adult patients with IDA and *H. pylori* infection are more likely to have a pattern of gastritis that involves the gastric corpus, with related decreases in gastric acid secretion and increases in intragastric pH that might impair iron absorption[110]. There are no comparable data in children.

Another hypothesized mechanism is that *H. pylori* might lead to IDA by sequestering and utilizing iron, thus

Table 3 Cross-sectional studies on the association between *Helicobacter pylori* infection and growth retardation

Author (yr)/country	Total No. of patients (*H. pylori* +)	Age (range, yr)	Diagnostic test	Conclusion
Perri *et al*[114] (1997)/Italy	216 (49)	3-14	Urea breath test	*H. pylori* infection was associated with growth delay, and poor socioeconomic status
Oderda *et al*[115] (1998)/Italy	134 with short stature (27)	5-13	Serology	No association with short stature
	134 controls (18)	5-13		
Quiñonez *et al*[116] (1999)/Guatemala	211 (107)	5-10	Serology	No association with height for age and nutritional status
Choe *et al*[90] (2000)/Korea	375 (63)	10-15	Serology	*H. pylori* infection accompanied by IDA, rather than *H. pylori* infection *per se*, was associated with delayed pubertal growth
Richter *et al*[117] (2001)/Germany	3315 (213)	5-7	Urea breath test	*H. pylori* infection was associated with growth delay
Ertem *et al*[118] (2002)/Turkey	327 (162)	3-12	Urea breath test	*H. pylori* infection was associated with short stature independently of poor living standards
Sood *et al*[119] (2005)/UK	257 (97)	-	Urea breath test	No association with height and weight z scores, after adjustment for socioeconomic status and ethnicity
Süoglu *et al*[120] (2007)/Turkey	70 (35)	4-16	Endoscopy	*H. pylori* infection and IDA had a significant effect on height z scores, after adjustment for economic status
Mohammad *et al*[121] (2008)/Egypt	286 (208)	6-15	Urea breath test	*H. pylori* infection affected both body weight and height
Soylu *et al*[122] (2008)/Turkey	108 with dyspepsia (57)	7-17	Endoscopy	No association with anthropometry. But, dyspeptic children had worse nutritional status compared to controls, regardless of *H. pylori* status
	50 healthy controls	8-17		
Cherian *et al*[123] (2009)/Australia	182 (149)	< 16	Stool antigen	No association with BMI or other anthropometric measures
Gulcan *et al*[124] (2010)/Turkey	181 with RAP (121)	6-15	Endoscopy, 181	RAP associated with gastric mucosal injury had a negative effect on BMI independent of *H. pylori* infection
	309 asymptomatic (110)	6-15	Serology, 309	RAP originating from *H. pylori* infection affected both BMI and linear growth

BMI: Body mass index; RAP: Recurrent abdominal pain; IDA: Iron deficiency anemia; *H. pylori*: *Helicobacter pylori*.

competing with the human host[107]. Ferrokinetic studies have suggested the diversion of iron to some extramedullary focus, hypothesized but not proven to be *H. pylori*-associated gastric infection[95]. Like many bacteria, *H. pylori* requires iron as a growth factor, and it possesses a 19-kDa iron-binding protein that resembles ferritin, which has been considered to play a role in storage of excess iron sequestered by the bacterium[111]. Another possible mechanism for IDA in *H. pylori*-infected subjects involves sequestration of iron in lactoferrin in the gastric mucosa, and uptake of iron by *H. pylori*. Lactoferrin is an iron-binding glycoprotein that is found in body fluids, and its secretion in the gastric mucosa seems to be influenced by some signal from *H. pylori*[112]. It appears that *H. pylori* then absorbs the iron from lactoferrin *via* a specific lactoferrin-binding protein that is expressed by *H. pylori*[107]. Lactoferrin levels in the gastric mucosa have been shown to be significantly higher in *H. pylori*-positive patients with IDA compared to those who are non-anemic *H. pylori*-negative, non-anemic *H. pylori*-positive, and *H. pylori*-negative with IDA[112].

We conclude that future work in this area is needed. Randomized, double-blind, and placebo-controlled trials of sufficient size and power should evaluate the long-term effect of *H. pylori* eradication in children with IDA, who are living in developing as well as in developed countries. Additionally, these studies should evaluate the effect of *H. pylori* treatment among different pediatric populations, such as those with and without concurrent gastrointestinal symptoms, and those with a wide spectrum of IDA severity.

Based on our present knowledge, children with a first episode of IDA and no complications should be initially treated with iron supplementation alone, irrespective of *H. pylori* status[2]. Eradication of *H. pylori* could be considered in cases that are refractory to iron supplementation and in the case of frequent relapses, assuming that other causes, such as celiac disease and inflammatory bowel disease, have been excluded[2,102]. Of particular interest is the work by Memeo *et al*[113] who have shown the frequent occurrence of duodenal intraepithelial lymphocyte expansion in individuals with *H. pylori* gastritis, and the considerable overlap of the intraepithelial lymphocyte counts as well as the distribution patterns with those described for celiac disease and other small bowel diseases.

GROWTH RETARDATION

The available evidence regarding *H. pylori* infection and its effect on growth in children is controversial. There have been many cross-sectional studies that point to either the presence or absence of such an association[90,114-124], as shown in Table 3.

The Italian cross-sectional study by Perri *et al*[114] suggests that *H. pylori* infection (as diagnosed by urea breath test) is associated with growth delay in older children, poor socioeconomic conditions, and household overcrowding. The findings by Perri *et al*[114] are consistent with the hypothesis that *H. pylori* infection is one of the environmental factors capable of affecting growth. The cross-section-

al study by Richter *et al*[117] of a large number of 5-7-year-old preschool and school children suggests that *H. pylori* infection (as diagnosed by urea breath test) is associated in German children with growth delay, growth retardation, or both, despite similar socioeconomic status between *H. pylori*-positive and -negative children. Likewise, Ertem *et al*[118] also have suggested that *H. pylori* is associated with short stature through mechanisms that are independent of poor living conditions.

Other investigators have suggested that growth suppression reported in children with *H. pylori* infection could be due to socioeconomic, genetic and environmental factors. In their retrospective chart review of the growth parameters of children with dyspepsia referred to the Regional Paediatric Gastroenterology unit in Manchester, United Kingdom, Sood *et al*[119] found that children with dyspepsia and *H. pylori* infection (as diagnosed by urea breath test) were shorter and lighter than patients with similar symptoms but no infection. Sood *et al*[119] concluded that the differences in anthropometry might have been due to socioeconomic and ethnic factors rather than *H. pylori* infection. Yet, in a cross-sectional study of Turkish dyspeptic children who were evaluated by endoscopic gastric biopsy for *H. pylori* infection, as well as a control group of age and sex cross-matched children, Soylu and Ozturk found that dyspeptic children with and without *H. pylori* infection had worse nutritional status compared to healthy controls[122]. The authors concluded that *H. pylori* infection as a major cause of dyspepsia might be considered to cause malnutrition secondary to decreased caloric intake associated with dyspepsia[122].

In a case-control study of children aged 5-13 years whose height was below the third centile, matched with children of the same age and sex whose height was above the 25th centile, Oderda *et al*[115] found that *H. pylori* (diagnosed by serologic methods) was not a risk factor for short stature, and that reduced growth was related to genetic determinants such as parental height and to mixed genetic and environmental factors such as birth weight. Low socioeconomic status was also relevant[115]. In a cross-sectional study of children aged 5-10 years who were attending an all-girl public school in inner Guatemala City, Quiñonez *et al*[116] investigated the effect of *H. pylori* infection (diagnosed by serologic methods) on the anthropometric nutritional parameters (weight-for-height and height-for-age). After controlling for sociodemographic variables, the authors did not find significant differences in the nutritional parameters between infected and uninfected children. In another cross-sectional study of Turkish children aged 4-16 years who underwent upper gastrointestinal endoscopy for RAP and dyspeptic complaints, Süoglu *et al*[120] found that the effect of *H. pylori* infection on mean SD scores of height for age was statistically insignificant after correction for breast feeding, IDA and socioeconomic level. In contrast, even after controlling for socioeconomic level, *H. pylori* infection remained the single and most important variable that had an effect on mean weight SD score. Among *H. pylori*-positive as well as -negative children[120], IDA had no significant effect on anthropometric measurements *per se*. However, when *H. pylori* and IDA were present, mean weight was found to be significantly lower than that of the *H. pylori*-negative patients without IDA[120].

There are also longitudinal studies that support the hypothesis that *H. pylori* infection might influence growth rate in children. Thomas *et al*[125] conducted two consecutive prospective, longitudinal cohort studies in Gambia, and found that, in both cohorts, children with early *H. pylori* colonization had lower values for both length- and weight-for-age Z scores than their peers in late infancy. No socioeconomic or demographic confounding variables were identified to explain this, and the weight deficit was no longer detectable when the children were aged 5-8 years. The authors concluded that *H. pylori* colonization at critical vulnerable ages might lead to malnutrition and growth retardation among infants in countries such as Gambia[125]. In the prospective, longitudinal study by Bravo *et al*[126], lower-middle class children from Colombia, in general good health, aged 1-5 years, who tested negative by urea breath test at baseline, were monitored over the following 2.5 years for anthropometric measurements every 2 mo, and for *H. pylori* by urea breath test every 4 mo. The authors found significant slowing of growth velocity in children infected with *H. pylori*, independent of socioeconomic variables or overcrowding[126]. Likewise, Mera *et al*[127] prospectively investigated in Colombian children, in general good health, aged 1-5 years, whether a newly acquired *H. pylori* infection affected height and weight of children within 16 mo, by performing breath tests and anthropometry every 2-4 mo. The authors observed that the impact of a new infection on height growth velocity was more pronounced right after the infection was diagnosed and slowly ebbed, and continued to be significant for up to 6 mo after infection, and became borderline significant at 8 mo after infection. No catch-up growth was evident in infected children, with crowding retarding linear growth. Compared with uninfected children, newly infected children also experienced a significant, but small, decrease in weight at the first follow-up visit, which was not statistically significant at 4 mo after infection. There was no catch-up in weight. The authors concluded that *H. pylori* caused a non-transient negative effect on height and weight in affected children, regardless of age at the time of infection[127].

Taken together, the results of these studies pointing to the presence or absence of an association between *H. pylori* and growth are subject to some potential limitations. First, most studies were cross-sectional or retrospective and therefore were unable to evaluate the possible effect of new infections on growth velocity. Second, the definition of socioeconomic status is complex and no set of parameters was fully descriptive in most studies. Third, some studies used serological methods to determine *H. pylori* infection. However, serology does not indicate whether there is active or past infection. Even in those patients who are treated and cured of their *H. pylori* infection, evidence of IgG antibodies may exist for several months

and possibly for years. Serological assays also have varying levels of sensitivity and specificity when used in different populations, particularly in children[128]. Fourth, in community-based studies, for example, in those that involve a blood specimen, participation rate is rarely more than 50%[116,129]. This might also be the case for studies that involve gastrointestinal endoscopy. As such, we have no information on the non-participating children in some of the above studies; thus, we cannot estimate if any bias was introduced in the results or the generalizability of their findings to other populations.

We conclude that future work in this area is needed to elucidate the importance of these factors. One would expect growth velocity to improve following *H. pylori* eradication, if the infection were the primary cause of growth suppression.

IDIOPATHIC THROMBOCYTOPENIC PURPURA

ITP is an autoimmune disease that is characterized by a low circulating platelet count caused by the destruction of antibody-sensitized platelets in the reticuloendothelial system[130]. The mechanisms that trigger the production of platelet autoantibodies remain poorly understood. Persistent thrombocytopenia for > 6 mo defines the chronic form of this disorder (cITP)[130]. Lately, eradication of *H. pylori* from the gastric mucosa has been associated with an improvement of cITP.

Several studies in adults have reported improved platelet counts in *H. pylori*-positive patients following standard triple *H. pylori* eradication therapy[131-140]. A meta-analysis of 13 cohort reports in adult cITP, with combined data from 193 patients, has indicated an overall response rate of 52% of patients after *H. pylori* eradication therapy[141]. A recent systematic review of 25 published studies, with combined data from 696 adult patients with cITP, has demonstrated an overall response in platelet count after *H. pylori* eradication in 50.3% of patients (95% CI: 41.6%-59.0%)[142]. Cohorts from Japan and Italy[132,134,138,140] have reported higher response rates than from other countries[143,144]. Several theories, including direct antigen mimicry between *H. pylori cagA* and platelet glycoprotein antigens, *H. pylori* binding to von Willebrand factor, and the immunomodulatory effect of antibiotics (e.g. macrolides) used in *H. pylori* eradication, have been proposed to explain the platelet response to anti-*H. pylori* therapy[139,141]. It has also been postulated that platelet autoantibodies might be produced by autoreactive clonal B cells that are induced by chronic immunological stimulus by *H. pylori*[141,145]. The relative toxicity profiles of triple therapy compared to standard ITP therapy certainly make eradication an attractive and generally safe option in adults. However, large controlled clinical trials of adult patients from various ethnic backgrounds are necessary to determine the response rate and mechanism of platelet response to *H. pylori* eradication therapy[141].

In children, the natural history of cITP is clearly different from that observed in adults. Spontaneous recovery occurs in one third of childhood cITP cases from several months to many years after their diagnosis, whereas only 5% of adults recover[130,146]. Thus, the effects of *H. pylori* eradication in childhood cITP could be different from those in adults. The issue of whether *H. pylori* eradication has a beneficial effect on the course of cITP in children has been the subject of few, apparently contradictory studies with small sample sizes[147-153]. Yet, the results of pediatric studies are difficult to compare because the prevalence of *H. pylori* infection and diagnostic methods vary among them[154]. Of note, in children with cITP, no studies have assessed, at the initial as well as follow-up visits, *H. pylori* status by upper gastrointestinal endoscopy.

In a study from Taiwan, Jaing *et al*[147] evaluated 22 cITP children, nine of whom were *H. pylori*-infected and were treated with a 1-wk course of triple therapy [including clarithromycin, amoxicillin, and proton pump inhibitors (PPIs)]. Of these nine patients, five (55.6%) were in complete or partial remission over a median of 16 mo follow-up, while four showed no improvement in platelet counts during 8-19 mo follow-up. In a study from the Netherlands, Neefjes *et al*[149] evaluated 47 children with cITP, three of whom were *H. pylori*-infected and were treated with a 2-wk course of the triple therapy mentioned above. Over a 6-mo follow-up, all three children achieved complete or partial remission. In a study from Iran, Hamidieh *et al*[150] evaluated 31 cITP children, four of whom were *H. pylori*-infected and were treated with a 2-wk course of the same triple therapy. None of the four patients achieved complete or partial remission after *H. pylori* eradication. In a study from Japan, Hayashi *et al*[148] evaluated 10 children with cITP, one of whom was *H. pylori*-infected and was treated with a 1-wk course of the same triple therapy. This child achieved complete remission throughout > 1 year of follow-up. In a study from Italy, Bisogno *et al*[151] evaluated 25 children with cITP, nine of whom were *H. pylori*-infected and had *H. pylori* eradication following 1-2 courses of 2 wk of the same triple therapy. Over 6-mo follow-up, of these nine patients, three had an increase in platelet count after eradication therapy, and one had complete remission and two had partial, transient remission followed by relapse a few months later. Over the same follow-up period, no significant increase in platelet count was seen in the other six eradicated patients. In the same study, Bisogno *et al*[151] also reported the platelet response in the 16 *H. pylori*-negative children with cITP. At 6-mo follow-up, two of these 16 patients achieved partial remission without any specific treatment. Yet, four of the *H. pylori*-negative children with cITP achieved spontaneous partial remission 1 year after diagnosis of *H. pylori* infection was excluded[151]. At latest follow-up, the remaining 10 *H. pylori*-negative children presented with a count above 50×10^9/L without any treatment. In another study from Italy, Loffredo *et al*[152] evaluated 39 children (median age, 136 mo) with cITP, eight of whom were *H. pylori*-infected and had *H. pylori* eradication following 1-3 courses of 2 wk of the same triple therapy. Over 1-year follow-up, none of the eight patients achieved complete or partial remission after *H. pylori* eradication[152].

Recently, Treepongkaruna *et al*[153] reported a multicenter randomized controlled trial of *H. pylori* eradication in 55 children with cITP. Of the 16 (29.1%) patients with cITP and *H. pylori* infection, seven were randomly treated with PPI-based triple therapy, while the remaining nine did not receive any specific treatment. If the first-line therapy failed to eradicate *H. pylori,* then second-line eradication therapy was used. Although eradication of *H. pylori* infection was successful in all patients in the treated group, the platelet recovery rate was not significantly different between the *H. pylori* treatment group and control group during the 6-mo period.

From the foregoing, whether antibiotic treatment of *H. pylori* infection should be considered in children with cITP is an unresolved question[154]. In conclusion, in view of the published evidence in children with cITP and the sporadic benefit of *H. pylori* eradication on the platelet response, the relationship between *H. pylori* and cITP in children warrants further investigation with large randomized controlled trials of sufficient size and power and across different ethnic populations[154].

ASTHMA AND ALLERGIC DISORDERS

In industrialized countries, the incidence of asthma, especially childhood asthma, has risen in recent years[155]. Conversely, in developed countries, the rate of acquisition of *H. pylori* has decreased substantially over recent decades[3,9]. The lack of early exposure to *H. pylori* has been suggested to be an important determinant of asthma risk in childhood[156]. In a recent retrospective, cross-sectional study, using data from 3327 participants, aged 3-19 years, *H. pylori* seropositivity was found to be inversely associated with onset of asthma before 5 years of age and current asthma in children aged 3-13 years[157]. *H. pylori* seropositivity also was inversely related to recent wheezing, allergic rhinitis, and dermatitis, eczema, or rash.

Allergic diseases and asthma are caused by exaggerated T-helper 2 (Th2)-biased immune response in genetically susceptible individuals[158]. A number of recent studies have indicated that regulatory T cells (Tregs) play an important role in controlling such Th2-biased responses. Impaired expansion of natural and/or adaptive Tregs is hypothesized to lead to the development of allergy and asthma[158]. Increased numbers of Tregs have been reported in the *H. pylori*-infected human gastric mucosa[159,160]. Thus, the absence of early exposure to *H. pylori* might cause the loss of a metabolically active lymphoid compartment in the stomach, including Tregs, which ultimately could affect the activity of T cells present in other mucosal and cutaneous sites[156,160]. Although this is an undoubtedly interesting theory, future prospective, longitudinal studies are needed to test the strength of the association between *H. pylori* status and asthma risk in children from developed and developing countries[161].

CONCLUSION

Many areas of active scientific inquiry have been reported in the recent literature about the disease spectrum of *H. pylori*. Several studies have demonstrated that *H. pylori* infection is not associated with specific symptomatology in children. Therefore, identification of children with *H. pylori*-associated gastritis on the basis of clinical presentation alone is not possible. Based on the best available evidence, testing for (and treating) *H. pylori* infection should be performed in children with endoscopically proven duodenal ulcer. Evidence from studies in adults supports the recommendation that testing for *H. pylori* should also be performed in children with a documented gastric ulcer. Endoscopy and biopsy are also recommended for children with persistent symptoms.

Pursuing *H. pylori* in asymptomatic children should be indicated for patients at increased risk of gastric cancer, for example, first-degree relatives of patients with gastric cancer, and individualized in populations at increased risk for gastric cancer, taking into consideration comorbid illness. Studies suggest that prevention of gastric cancer is possible in infected individuals with no precancerous lesions. Therefore, children might well be the group to target in an effort to prevent future development of gastric cancer.

Although dyspepsia and *H. pylori* infection are common in the general population, current data in the literature regarding a causal association between *H. pylori* gastritis and dyspepsia are conflicting. It is uncertain whether eradication of the infection leads to an improvement of symptoms. Randomized, placebo-controlled, double-blind trials with minimal loss to follow-up, strict group definition, and standardized and validated outcome measures are needed.

There is no compelling evidence to support routine testing in children with cITP, poor growth, and GERD. In children with refractory IDA, where other causes have been ruled out, testing for (and treating) *H. pylori* infection can be considered. Prospective, longitudinal studies are needed to test the strength of the newly reported association between *H. pylori* status and asthma risk in children. In the absence of these studies, there is little call to leave *H. pylori* infection untreated in patients with asthma and allergy. We believe that *H. pylori* eradication is strongly beneficial for curing peptic ulcer disease and gastric lymphoma and for prevention of gastric cancer, as well as other diseases that are putatively linked to infection, and it must be done in *H. pylori*-infected patients, whether or not they have asthma.

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ORIGINAL ARTICLE

Prognostic relevance of *β-catenin* expression in T2-3N0M0 esophageal squamous cell carcinoma

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Abstract

AIM: To study the expression of *β-catenin* in esophageal squamous cell carcinoma (ESCC) at stage T2-3N0M0 and its relation with the prognosis of ESCC patients.

METHODS: Expression of *β-catenin* in 227 ESCC specimens was detected by immunohistochemistry (IHC). A reproducible semi-quantitative method which takes both staining percentage and intensity into account was applied in IHC scoring, and receiver operating characteristic curve analysis was used to select the cut-off score for high or low IHC reactivity. Then, correlation of *β-catenin* expression with clinicopathological features and prognosis of ESCC patients was determined.

RESULTS: No significant correlation was observed between *β-catenin* expression and clinicopathological parameters in terms of gender, age, tumor size, tumor grade, tumor location, depth of invasion and pathological stage. The Kaplan-Meier survival curve showed that the up-regulated expression of *β-catenin* indicated a poorer post-operative survival rate of ESCC patients at stage T2-3N0M0 (P = 0.004), especially of those with T3 lesions (P = 0.014) or with stage ⅡB diseases (P = 0.007). Multivariate analysis also confirmed that *β-catenin* was an independent prognostic factor for the overall survival rate of ESCC patients at stage T2-3N0M0 (relative risk = 1.642, 95% CI: 1.159-2.327, P = 0.005).

CONCLUSION: Elevated *β-catenin* expression level may be an adverse indicator for the prognosis of ESCC patients at stage T2-3N0M0, especially for those with T3 lesions or stage ⅡB diseases.

Key words: Esophageal squamous cell carcinoma; *β-catenin*; Prognosis; Receiver operating characteristic curve; Immunohistochemistry

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INTRODUCTION

Esophageal carcinoma, an aggressive tumor with a poor prognosis, is one of the most common malignant tumors in Asia, especially in certain areas of China, and South

America[1]. Esophageal squamous cell carcinoma (ESCC) accounts for over 90% of all esophageal cancers worldwide[2]. Despite advances in imaging technologies enabling earlier diagnosis of ESCC, surgery and response rates of radiotherapy and chemotherapy, the clinical outcome of ESCC patients remains unsatisfactory. Even in the developed world, more than 85% of ESCC patients die within 2 years after its diagnosis[3]. In China, the esophageal cancer mortality rate ranks fourth of cancer-related deaths[4]. Thus, improvement in the efficacy of ESCC treatment is a major public health goal. New modalities based on a better understanding of the ESCC biology are indispensable. Since the classical staging criteria fail to differentiate the prognostic characteristics of ESCC patients adequately, molecular tumor analysis may provide a necessary means for defining the prognosis of ESCC patients. In order to further improve the survival rate of ESCC patients, it is essential to identify the relevant biomarkers with adverse prognostic significance, and to modify the therapeutic strategies for individual patients according to their molecular tumor status.

β-catenin is an 88kDa versatile protein that has at least two different cellular functions[5-7]. First, *β-catenin* is an important structural component of both normal epithelium and malignant cells. Together with a structurally homologous *γ-catenin*, *β-catenin* participates in cell-cell and cell-matrix adhesion by binding to the intracellular domain of *E-cadherin*, a homotypic cell-to-cell interaction molecule ubiquitously expressed in epithelial cells[6,8,9]. In addition, these catenins play an important role in cell polarity by binding to the actin filament network of cytoskeleton through *a-catenin* as a linker[6,8]. Unlike other known catenins, *β-catenin* is also a key mediator in the Wingless/Wnt signal transduction pathway[7,8,10]. In cytoplasm of normal cells, the amount and location of *β-catenin* are controlled exquisitely through its association with the adenomatous polyposis coli (*APC*) tumor-suppressor gene product, a scaffolding protein *Axin*, and a glycogen synthetase kinase (*GSK-3β*) enabling phosphorylation and degradation of free *β-catenin*[6-10]. Once located in nuclei, *β-catenin* can act as a transcription factor by serving as a coactivator of the lymphoid enhancer factor/TCF family of DNA-binding proteins[10]. Activation of Wnt signaling involves the inhibition of catenin degradation by proteasomes, resulting in its cytoplasmic and nuclear accumulation and transcriptional activation of the target gene[5-7,10]. It is believed that *β-catenin* integrity impairment -related intracellular network may be closely associated with the dedifferentiation, hyperproliferation, invasion and metastatic potential of malignancy[10,11]. This biomarker has thereby been extensively studied in a variety of neoplasms, such as hepatocellular carcinoma[12], colorectal carcinoma[13], gastric cancer[14,15], pancreatic cancer[16], ovarian cancer[17], lung cancer[18-20], breast cancer[21], nasopharyngeal carcinoma[22], prostate cancer[23] and even lymphoma[24], with regard to its potential role as a prognostic factor in cell polarity. The present findings in ESCC are controversial in the literature[25-28]. The role of *β-catenin* in development of ESCC and its prognostic significance remain to be defined. In the present study, the expression level of *β-catenin* was measured in specimens from a relatively homogeneous cohort of ESCC patients with no lymph node involved, which was correlated with the clinical outcome of ESCC patients.

MATERIALS AND METHODS

Patients and tissue samples

The study was approved by the Ethics Committee of Sun Yat-Sen University Cancer Center. A total of 227 consecutive patients with node-negative ESCC at stage ⅠB-ⅡB who underwent curative surgery from January 1993 to August 2004 were enrolled in this study. *β-catenin* expression level was measured in resected specimens with immunohistochemistry (IHC). Patients were followed up prospectively and their survival data were recorded through October 2009. The inclusion criteria were patients with histopathologically-proven ESCC, those with esophageal cancer at T2-3N0M0 based on the seventh edition of the American Joint Committee on Cancer staging system[29], those with at least 15 lymph nodes to be removed for pathological evaluation, those at the age of at least 18 years, those with no evidence of metastatic disease as determined by history, physical examination, and blood chemistry analysis or routine computed tomography, those with no history of adjuvant therapy. Patients with a history of previously treated cancer other than basal or squamous cell carcinoma of the skin, preoperative chemotherapy and/or radiotherapy, or with unknown causes of death in follow-up were excluded from the study.

Immunohistochemistry

β-catenin (CAT-5H10, Fuzhou Maxim Inc., Fuzhou, Fujian province, China) was diluted at 1:100. ESCC tissue was cut into 4 μm-thick paraffin sections, which were stained with immunoperoxidase. The sections were deparaffinized in xylene, hydrated prior to antigen retrieval by microwaving in sodium citrate buffer (pH 6.0), and incubated with a peroxidase block followed by primary antibody. After washed with PBS, the sections were incubated first with secondary antibody followed by 3,3'-diaminobenzidine, and then counterstained with hematoxylin (Hematoxylin 7211; Richard-Allen Scientific, Kalamazoo, Michigan, USA). The peroxidase block, secondary antibody and 3,3'-diaminobenzidine were all obtained from the Dako-Cytomation EnVision System (Glostrup, Denmark).

Immunohistochemical scoring

β-catenin was scored with IHC using a semi-quantitative system as previously described[30,31]. Each section was assigned a score and the score of tumor cell staining was multiplied by the score of staining intensity. Tumor cell staining was scored using a semi-quantitative six-category grading system: 0 = no tumor cell staining, 1 = 1%-10% of tumor cells staining, 2 = 11%-25% of tumor cells staining, 3 =

26%-50% of tumor cells staining, 4 = 51%-75% of tumor cells staining, 5 = over 75% of tumor cells staining. Stain intensity was scored using a semi-quantitative four-category grading system: 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining. Two experienced pathologists blinded to the clinical follow-up data independently scored the 400 ESCC samples including the cases used in this study. The complete score agreement of the two pathologists was 87% of all cases, indicating that the scoring method is reasonably reproducible. A third blinded pathologist intervened and evaluated the patients with different IHC scores. If the third pathologist agreed with one of the previous scores, it was used for analysis. The three pathologists were asked to reach an agreement on the cases from which three different scores were obtained.

Selection of cut-off scores

Cut-off scores for *β-catenin* expression were selected based on receiver operating characteristic (ROC) curve analysis. ROC curve was plotted for the outcome of ESCC patients under study by calculating the sensitivity and specificity on its points. The score closest to the points (0.0, 1.0) on the curve with a maximum sensitivity and specificity was selected as the cut-off score leading to the greatest number of tumors classified with or without clinical outcome. The area under the ROC curve was calculated to estimate the discriminatory power of *β-catenin* over the entire range of scores for overall survival (OS) rate of ESCC patients. The ROC curve was generated and analyzed using the MedCalc statistical software package 11.0.1 (MedCalc Software bvba, Belgium).

Statistical analysis

Association between categorical variables was analyzed by χ^2 test. Survival curves were calculated with the Kaplan-Meier method and compared by the log-rank test. Time of death was calculated from the date of surgery to the date of death. The time variable was censored on the date of last follow-up of event-free subjects. Multivariate analysis of prognostic factors was performed using the Cox's regression model. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS 13.0 for Windows software system (SPSS Inc., Chicago, IL).

RESULTS

Characteristics of patients and expression of β-catenin

The demographic and clinicopathological parameters of ESCC patients included in this study are listed in Table 1. Various intensities of positive *β-catenin* reaction were detected in cytoplasm and membrane of cancer cells (Figure 1). According to the ROC curves for OS rate, a threshold value of 1.3333 was the optimal point for maximum sensitivity and specificity, and selected as the cut-off score (Figure 2). The 227 ESCC specimens were then catego-

Table 1 Demographic and clinicopathological parameters of esophageal squamous cell carcinoma patients included in this study *n* (%)

Demographic and clinicopathological parameters	Patients with ESCC
Sex	
Male	165 (72.7)
Female	62 (27.3)
Age (yr)	
Median	58
Range	33-77
Tumor size (cm)	4.92 ± 2.046
Tumor grade	
Grade 1	57 (25.1)
Grade 2	114 (50.2)
Grade 3	56 (24.7)
Tumor location	
Upper	25 (11.0)
Middle	152 (67.0)
Lower	50 (22.0)
Depth of invasion	
T2	88 (38.8)
T3	139 (61.2)
AJCC staging system (7th ed)	
Ⅰ B	12 (5.3)
Ⅱ A	83 (36.6)
Ⅱ B	132 (58.1)

ESCC: Esophageal squamous cell carcinoma; AJCC: American Joint Committee on Cancer.

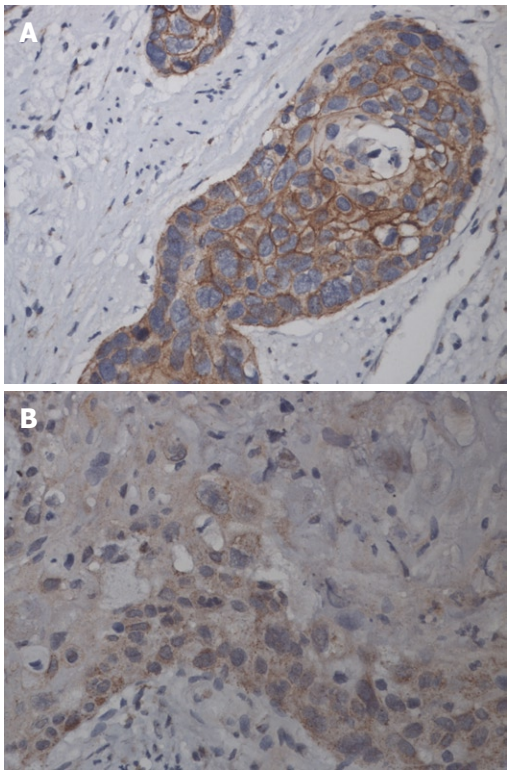

Figure 1 Expression of *β-catenin* in esophageal squamous cell carcinoma tissue samples (magnification × 400). A: Immunohistochemical staining of *β-catenin* in cytoplasm and membrane of cancer cells (immunohistochemistry score: 15); B: Immunohistochemical staining of *β-catenin* in cytoplasm of cancer cells (immunohistochemistry score: 3).

Table 2 *β-catenin* expression in esophageal squamous cell carcinoma patients *n* (%)

Parameters of ESCC patients	*n*	Expression of *β-catenin*		*P*-value
		Low	High	
Sex				
Male	165	57 (34.5)	108 (65.5)	0.354
Female	62	26 (41.9)	36 (58.1)	
Age (yr)				
≤ 60	138	49 (35.5)	89 (64.5)	0.778
> 60	89	34 (38.2)	55 (61.8)	
Tumor size (cm)				
≤ 5.0	155	54 (34.8)	101 (65.2)	0.461
> 5.0	72	29 (40.3)	43 (59.7)	
Tumor grade				
Grade 1	57	17 (29.8)	40 (70.2)	
Grade 2	114	44 (38.6)	70 (61.4)	0.473
Grade 3	56	22 (39.3)	34 (60.7)	
Tumor location				
Upper	25	11 (44.0)	14 (56.0)	
Middle	152	52 (34.2)	100 (65.8)	0.545
Lower	50	20 (40.0)	30 (60.0)	
Depth of invasion				
T2	88	34 (38.6)	54 (61.4)	0.672
T3	139	49 (35.3)	90 (64.7)	
AJCC staging system (7th ed)				
Ⅰ B	12	3 (25.0)	9 (75.0)	
Ⅱ A	83	31 (37.3)	52 (62.7)	0.694
Ⅱ B	132	49 (37.1)	83 (62.9)	

ESCC: Esophageal squamous cell carcinoma; AJCC: American Joint Committee on Cancer.

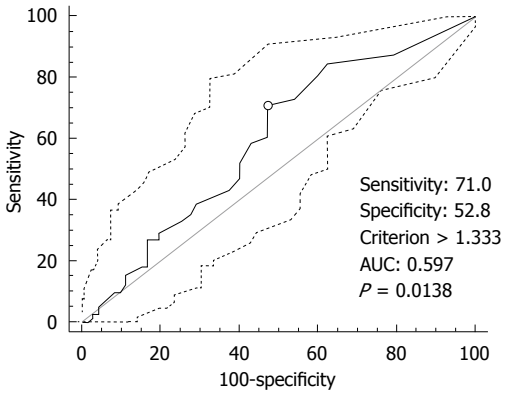

Figure 2 Receiver operating characteristic curve analysis of *β-catenin* and selection of cut-off score.

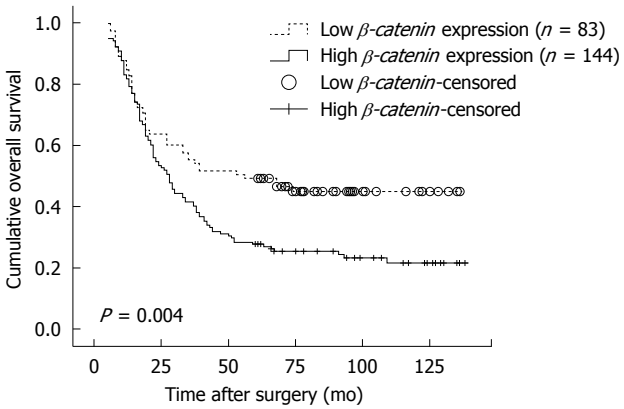

Figure 3 Kaplan-Meier survival curves for patients with esophageal squamous cell carcinoma at stage T2-3N0M0 according to *β-catenin* expression.

rized into high and low *β-catenin* expression groups. The expression level of *β-catenin* was up-regulated in 144 cases (63.4%) and down-regulated in 83 cases (36.6%).

Correlation between β-catenin expression and clinicopathological features

The correlation between *β-catenin* expression in and clinicopathological features of ESCC patients are shown in Table 2. No significant correlation was identified between *β-catenin* expression and any clinicopathological parameters, including gender, age, tumor size, tumor grade, tumor location, depth of invasion and pathological stage based on the seventh edition of AJCC staging system[29].

β-catenin expression and survival rate

At the time of data analysis (October 2009), 72 patients (31.7%), with a median follow-up time of 32 mo (range 5-138 mo), remained alive and 155 patients (68.3%) died. The overall 1-, 3- and 5-year survival rates for the patients were 58%, 39%, and 33%, respectively.

The Kaplan-Meier survival curves (Figure 3) showed that the post-operative survival rate of patients with a low *β-catenin* expression level was significantly higher than that of those with a high *β-catenin* expression level ($P = 0.004$). Further stratified analysis split by depth of invasion (Figure 4) showed that the expression of *β-catenin* had a statistically significant influence on the survival rate of patients with T3 diseases ($P = 0.014$) rather than on the survival rate of those with T2 lesions ($P = 0.145$). Furthermore, the stratified analysis split by pathological stage based on the new staging system (Figure 5) revealed that *β-catenin* expression had a significant influence on the prognosis of patients with ESCC at stage ⅡB ($P = 0.007$) but not on the prognosis of those with ⅡA diseases ($P = 0.253$). Patients with ESCC at stage ⅠB were not included in this analysis due to a small sample size.

Factors involved in OS rate of ESCC patients were identified using the Cox proportional hazards model (Table 3). Univariate analysis showed that tumor grade, depth of invasion and *β-catenin* expression were found to be the significant prognostic indicators for the OS rate of ESCC patients, and thereby selected as the parameters to be included in the same Cox regression model. Further multivariate analysis also confirmed that *β-catenin* expression (relative risk = 1.642, 95% CI: 1.159-2.327, $P = 0.005$), tumor grade (relative risk = 1.549, 95% CI: 1.095-2.190, $P = 0.013$) and depth of invasion (relative risk = 1.493, 95% CI: 1.066-2.089, $P = 0.020$) were the independent prognostic factors for the OS rate of ESCC patients.

DISCUSSION

To date, several IHC studies have been performed in order

Table 3 Univariate and multivariate analysis of overall survival rate of esophageal squamous cell carcinoma patients with Cox proportional hazards model

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI	*P*-value
Age (yr)						
≤ 60 *vs* > 60	1.023	0.740-1.414	0.889			
Gender						
Male *vs* female	0.692	0.474-1.009	0.056			
Tumor size (cm)						
≤ 5 *vs* > 5	0.987	0.704-1.386	0.942			
Grade						
G1 *vs* G2 and 3	1.498	1.060-2.116	0.022[a]	1.549	1.095-2.190	0.013[a]
Tumor location						
Upper and middle *vs* lower	0.954	0.657-1.385	0.804			
Depth of invasion						
T2 *vs* T3	1.461	1.045-2.043	0.026[a]	1.493	1.066-2.089	0.020[a]
AJCC staging system (7th ed)						
ⅠB and ⅡA *vs* ⅡB	1.192	0.865-1.643	0.284			
β-catenin						
Low *vs* high	1.644	1.161-2.329	0.005[b]	1.642	1.159-2.327	0.005[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* univariate analysis. AJCC: American Joint Committee on Cancer; CI: Confidence interval.

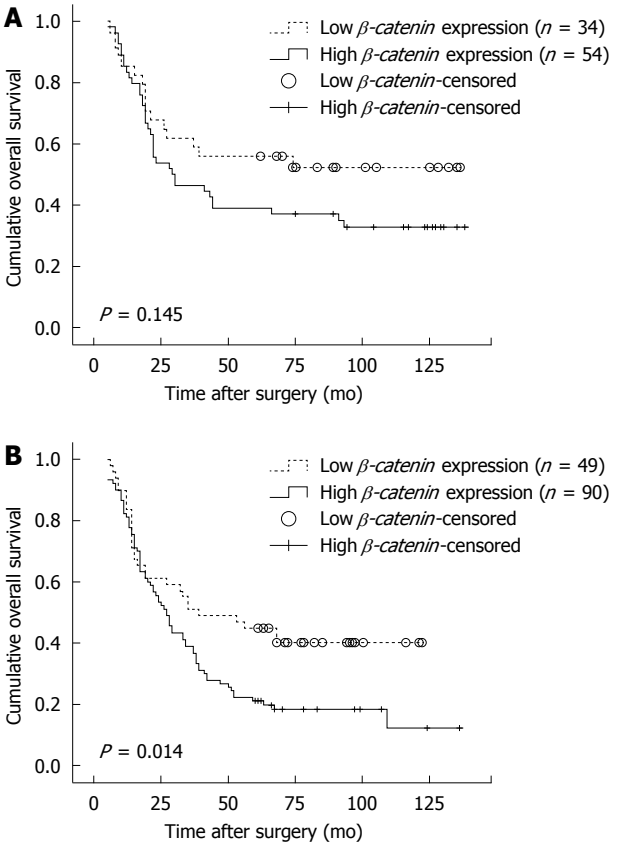

Figure 4 Kaplan-Meier survival curves for patients with esophageal squamous cell carcinoma according to *β-catenin* expression. A: Correlation between *β-catenin* expression and post-operative survival rate of patients with T2 lesions; B: Correlation between *β-catenin* expression and post-operative survival rate of patients with T3 lesions.

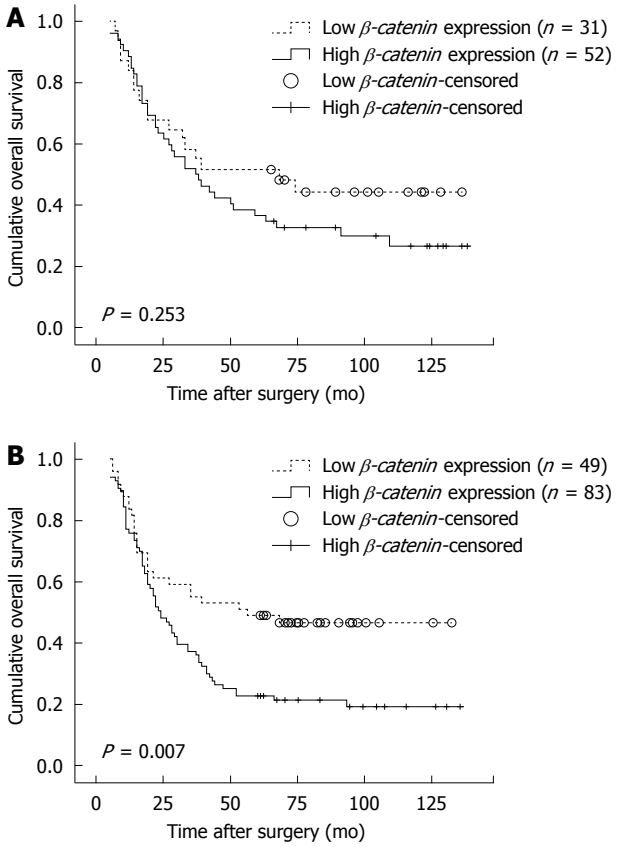

Figure 5 Kaplan-Meier survival curves for patients with esophageal squamous cell carcinoma stratified for pathological stage according to *β-catenin* expression. A: Correlation between *β-catenin* expression and post-operative survival rate of patients with ⅡA diseases; B: Correlation between *β-catenin* expression and post-operative survival rate of patients with ⅡB diseases.

to elucidate the role of *β-catenin* in ESCC, but the current findings in terms of its expression pattern and potential involvement in formation and progression of ESCC are contradictory in the literature[25-28]. One problem faced by researchers is the determination of tumor immunohistochemical positivity for *β-catenin* which is clinically and biologically relevant. Previous studies have applied different scoring systems in predetermination of cut-off scores

which might be set arbitrarily[30-33]. A lack of consistent, widely applicable methodology may have been primarily responsible for the contradictory results of these studies evaluating *β-catenin* and its prognostic value in ESCC patients. Therefore, our study used a reproducible scoring method which takes both staining percentage and intensity into account, and the cut-off score was selected based on ROC curve analysis, so that the trade-off between sensitivity and specificity was the smallest, leading to the greatest overall number of correctly classified tumors with and without clinical outcome.

In the present study, univariate and multivariate analyses showed that high expression level of *β-catenin* in completely resected samples from patients with ESCC at stage T2-3N0M0 was significantly correlated with the worse post-operative survival rate of ESCC patients, which is consistent with the reported findings[27]. Krishnadath *et al*[34] reported that low expression level of *β-catenin* is significantly correlated with the poor prognosis of esophageal adenocarcinoma patients, especially at early-stage. It has been shown that the higher the expression level of *β-catenin* is, the better the outcome of esophageal adenocarcinoma patients is, and the *β-catenin* expression level is higher in more invasive tumors than in superficial tumors[35]. However, Zhao *et al*[26] and Lin *et al*[28] demonstrated that *β-catenin* expression does not imply more aggressive malignant behaviors of ESCC or predict the poor prognosis of ESCC patients. Krishnadath *et al*[34] and Osterheld *et al*[35] showed a different histological type of tumor (adenocarcinoma) when they analyzed these contradictory results. In addition, esophageal carcinoma at stages I -Ⅳ was involved in the studies[26,28,34,35], suggesting that different therapeutic strategies including adjuvant or neoadjuvant chemotherapy and radiotherapy for more advanced disease may introduce confounding factors affecting the application of molecular analysis in assessing the prognosis of ESCC patients.

β-catenin protein not only serves as a pivotal component of *E-cadherin/catenin* complex which participates in cell-cell and cell-matrix adhesion[6,8,9], but also as a key mediator in the Wingless/Wnt signal transduction pathway[5,7], indicating that disruption of *E-cadherin/catenin* complex or physical and functional loss of *β-catenin* protein can lead to loosening of cell-cell contact and promote tumor invasion and metastasis. On the other hand, *β-catenin* can be oncogenically activated either by direct gene mutation[36] and inactivation of the *APC* tumor suppressor[37], or by activation of the Wingless/Wnt signal transduction pathway[38], thus resulting in post-translational stabilization of *β-catenin* protein. Excess cytoplasmic accumulation of *β-catenin* protein can then increase the influx of this molecule into nuclei, leading to over-expression of tumor-promoting genes, such as *cyclin-D1* and *c-myc*[5,6], and promote cell mitosis and growth[39,40]. Therefore, either up-regulated or down-regulated expression of *β-catenin* contributes to invasive and metastatic potentials of esophageal cancer. Obviously, the question is which physiopathological process takes the advantage in different circumstances, such as different histological types or different pathological stages. Further analysis of the role of *β-catenin* gene and its products in formation and progression of esophageal carcinomas may provide a better understanding of this pathogenic process.

In this study, further stratified analysis split by pathological stage and depth of invasion showed that *β-catenin* exhibited its effect on the prognosis of patients with ESCC at stage ⅡB or with T3 lesions, indicating that this biomarker is more valuable in predicting the outcome of ESCC patients at advanced stages, which is consistent with the findings in other studies[2,22]. Further study is needed to verify this trend. In this study, no significant correlation was found between *β-catenin* expression and prognostic parameters, including tumor grade, tumor location, depth of invasion and pathological stage. Multivariate survival analysis of all potential prognostic variables also confirmed that *β-catenin* was an absolutely independent prognostic factor, which is in accordance with the reported findings[25,28]. However, other studies showed that *β-catenin* is significantly correlated with the accepted prognostic parameters of ESCC[26,27,41]. Further study with a large sample size is needed to obtain a clearer picture.

In conclusion, elevated *β-catenin* expression level is an adverse prognostic factor for ESCC patients at stage T2-3N0M0, especially for those with T3 lesions or with stage ⅡB diseases. However, further study with a larger cohort of patients is required to verify this observation, especially in view of the contradictory results.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC), an aggressive tumor with a poor prognosis, is one of the most common malignant tumors in Asia, especially in certain areas of China. Despite advances in early diagnosis and therapies, the clinical outcome of ESCC patients remains unsatisfactory. Since the classical staging criteria fail to differentiate prognostic characteristics of ESCC patients adequately, many efforts have been made to identify relevant biomarkers with adverse prognostic significance, and to modify therapeutic strategies for individual patients. *β-catenin*, as a prognostic factor for ESCC, has been extensively studied in a variety of neoplasms. However, the exact role of *β-catenin* and its prognostic significance in ESCC remain to be defined.

Research frontiers

β-catenin protein not only serves as a pivotal component of *E-cadherin/catenin* complex which participates in cell-cell and cell-matrix adhesion, but also as a key mediator in the Wingless/Wnt signal transduction pathway. The hotspot in molecular tumor analysis of *β-catenin* is whether *β-catenin* involves and how it involves in the formation and progression of esophageal carcinoma.

Innovations and breakthroughs

Previous immunohistochemistry studies showed contradictory results in *β-catenin* expression pattern and its prognostic value for ESCC, due to lack of consistent, widely applicable methods. Therefore, the present study used a reproducible scoring method which takes both staining percentage and intensity into account, and the cut-off score was selected based on receiver operating characteristic (ROC) curve analysis so that the trade-off between sensitivity and specificity was the smallest, leading to the greatest overall number of correctly classified tumors with and without clinical outcome. This is the first study to evaluate *β-catenin* expression in ESCC patients with this novel method, showing that elevated *β-catenin* expression is an adverse prognostic factor for ESCC patients at stage T2-3N0M0, especially for those with T3 lesions or stage ⅡB diseases.

Applications

The novel methods used in this study can be applied in treatment of ESCC patients at stage T2-3N0M0.

Terminology

ROC curve analysis: In signal detection theory, a ROC curve is a graphical plot of the sensitivity, or true positivity *vs* (1-specificity), or false positivity for a binary classifier system as its discrimination threshold is varied. The ROC curve can also be represented equivalently by plotting the fraction of true positivity (TPR = true positive rate) *vs* the fraction of false positivity (FPR = false positive rate). ROC curve analysis provides tools to select possibly optimal models and to discard suboptimal ones independently from (and prior to specifying) the cost context or the class distribution. ROC curve analysis is related in a direct and natural way to cost/benefit analysis of diagnostic decision making. *β-catenin*: *β-catenin* protein was originally identified as a component of adherence junction, a multi-protein complex supporting tight cell-cell contacts in the presence of extracellular calcium. However, *β-catenin* also plays a key role in the Wnt signaling transduction pathway.

Peer review

The authors studied the expression of *β-catenin* in ESCC at stage T2-3N0M0 and its prognostic significance by analyzing the expression of *β-catenin* in 227 ESCC specimens with IHC and ROC curve analysis to select the cut-off score for high or low IHC reactivity. Then, they correlated the *β-catenin* expression with clinicopathological features of ESCC patients and its relation with the prognosis of ESCC patients. No significant correlation was observed between *β-catenin* expression in and clinicopathological parameters of ESCC patients, but multivariate analysis confirmed that *β-catenin* was an independent prognostic factor for the overall survival rate of ESCC patients at stage T2-3N0M0. The manuscript reads nicely and is easy to follow. Tables are legible and easy to understand. I believe that the method used in this study is plausible and the conclusion is well supported by the data. This manuscript adds to the current knowledge on this topic and it is a pleasure to read it.

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ORIGINAL ARTICLE

Classification of histological severity of *Helicobacter pylori*-associated gastritis by confocal laser endomicroscopy

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Abstract

AIM: To classify the histological severity of *Helicobacter pylori* (*H. pylori*) infection-associated gastritis by confocal laser endomicroscopy (CLE).

METHODS: Patients with upper gastrointestinal symptoms or individuals who were screened for gastric cancer were enrolled in this study. Histological severity of *H. pylori* infection-associated gastritis was graded according to the established CLE criteria. Diagnostic value of CLE for histological gastritis was investigated and compared with that of white light endoscopy (WLE). Targeted biopsies from the sites observed by CLE were performed.

RESULTS: A total of 118 consecutive patients with *H. pylori* infection-associated gastritis were enrolled in this study. Receiver operating characteristic curve analysis showed that the sensitivity and specificity of CLE were 82.9% and 90.9% for the diagnosis of *H. pylori* infection, 94.6% and 97.4% for predicting gastric normal mucosa, 98.5% and 94.6% for predicting histological active inflammation, 92.9% and 95.2% for predicting glandular atrophy, 98.6% and 100% for diagnosing intestinal metaplasia, respectively. Post-CLE image analysis showed that goblet cells and absorptive cells were the two most common parameters on the CLE-diagnosed intestinal metaplasia (IM) images ($P < 0.001$). More histological lesions of the stomach could be found by CLE than by WLE ($P < 0.001$).

CONCLUSION: CLE can accurately show the histological severity of *H. pylori* infection-associated gastritis. Mapping IM by CLE has a rather good diagnostic accuracy.

Key words: Confocal laser endomicroscopy; *Helicobacter pylori*; Gastritis; Classification; Histology

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a well accepted major etiological factor for gastric diseases, such as chronic gastritis, peptic ulcer and gastric carcinoma[1-3]. Therefore, endos-

copists must determine the presence of *H. pylori* infection in the stomach during or after endoscopy. The updated Sydney system for classification of gastritis has established the association between *H. pylori* infection and histological evidence of gastritis[4]. Gastric atrophy especially accompanying intestinal metaplasia (IM), which is the severest stage of gastritis and has a high risk for gastric cancer, is closely associated with *H. pylori* infection[5]. Although the Sydney system has been widely used, multiple biopsies are rarely performed for gastritis evaluation but only for suspected lesions of cancer in clinical practice, thus leading to omission of some precancerous lesions which are difficult to find by white light endoscopy (WLE)[6]. Therefore, *in vivo* detection of *H. pylori* infection and its related complications by endoscopy is a simple, noninvasive and inexpensive procedure. *H. pylori*-induced gastritis must be evaluated and graded especially in Eastern countries because of a high prevalence of *H. pylori* infection and a high incidence of gastric cancer[7]. Furthermore, it can facilitate the clinical assessment and timely treatment of gastritis. The association between *H. pylori* infection and endoscopic findings has been extensively studied using modern endoscopic techniques such as magnification endoscopy and narrow-band imaging[8-10].

Confocal laser endomicroscopy (CLE) can detect gastrointestinal diseases, such as Barrett esophagus, gastric carcinoma and colonic neoplasia[11,12]. CLE can observe real-time histological-like cellular and subcellular conditions of gastric mucosal layer at the magnification × 500-1000. It was reported that acriflavine-aided CLE can observe *H. pylori in vivo*[13]. In addition, gastric pit patterns of various gastropathies have been accurately classified and IM has been studied by CLE[14,15]. However, no systematic data are available on the CLE characteristics of chronic gastritis and the classification of chronic gastritis has not been investigated by CLE.

In this study, the CLE features of gastritis mainly caused by *H. pylori* infection were compared to evaluate the accuracy of CLE in diagnosing *H. pylori* infection and the severity of gastritis.

MATERIALS AND METHODS

Patients

Consecutive patients with upper gastrointestinal symptoms or individuals who were screened for gastric carcinoma, admitted to our hospital from June to November 2009, were enrolled in this study. The following patients were excluded from the study, including those who received proton pump inhibitors, antibiotics, or bismuth subsalicylate in the previous 6 wk, those with a history of using nonsteroidal anti-inflammatory drugs and medication for *H. pylori* infection, those undergone stomach surgery, those with systematic diseases or known gastric carcinoma, pregnant or breast-feeding females, those who did not give their informed consent or had an allergy to fluorescein. All participants gave their written informed consent before endoscopy. The study was approved by the Ethics Committee of Qilu Hospital.

CLE system

A confocal laser endomicroscope (Pentax ISC-1000, Pentax, Tokyo, Japan) was used in this study. It is novel digestive endoscope with a confocal laser microscope integrated into the distal tip, can realize a histological-like examination during routine endoscopy and accurately diagnose gastrointestinal diseases at the magnification × 1000. CLE was performed to scan the gastric mucosa from the top layer to 250 μm beneath the surface. The CLE and white-light endoscopy (WLE) images were captured and stored.

CLE procedure

CLE was performed by an endoscopist experienced with the system. All patients received oral chymotrypsin (20000 U) to eliminate the slime layer of the stomach for better visualization. One mL of 2% fluorescein (a contrast agent) was intravenously injected before endoscopy. After a first WLE of the stomach, 10 mL of 10% fluorescein was intravenously injected, and CLE images could be seen after a few seconds. Five standardized sites (2 from the lesser and greater curvatures of the antrum about 2-3 cm near the pylorus, 2 from the middle portion of the lesser and greater curvatures of the corpus about 8 cm from the cardia, and 1 from the angulus), as recommended by the updated Sydney system, were examined separately on CLE images. Shallow-deep CLE images were captured at each site and stored as digital files for further analysis. At the standardized locations, real-time image assessment and targeted biopsies were performed by CLE for histopathology. For *H. pylori* testing, 2 specimens were taken from the greater curvature of the antrum and corpus, respectively. If necessary, other scanning and biopsies were performed for lesions such as those with color changes or erosions, polyps, ulcers, or abnormal folds.

CLE classification of H. pylori infection-associated gastritis severity

In our study, the severity of gastritis was divided into 6 levels by CLE: normal mucosa, *H. pylori*-associated active inflammation (3 levels), glandular atrophy and IM.

CLE criteria for normal gastric mucosa and *H. pylori*-associated active inflammation: The published CLE classification of gastric pit patterns was used for one part of our classification of *H. pylori*-associated active inflammation[14]. In addition, a new marker, fluorescein leakage, was introduced to define the active inflammation in stomach on CLE images (Figure 1). Each of the 5 standardized sites was assigned to a confocal gastritis score (CGS) and a corresponding histologic gastritis score (HGS).The CGS for normal mucosa, and mild, moderate and marked active inflammation was 0, 1, 2 and 3, respectively. The severity of histological activity and chronic inflammation was also defined as normal, mild, moderate and marked with a score of 0-3, respectively. Finally, the mean CGS and HGS (mCGS and mHGS) were calculated for specimens from each stomach.

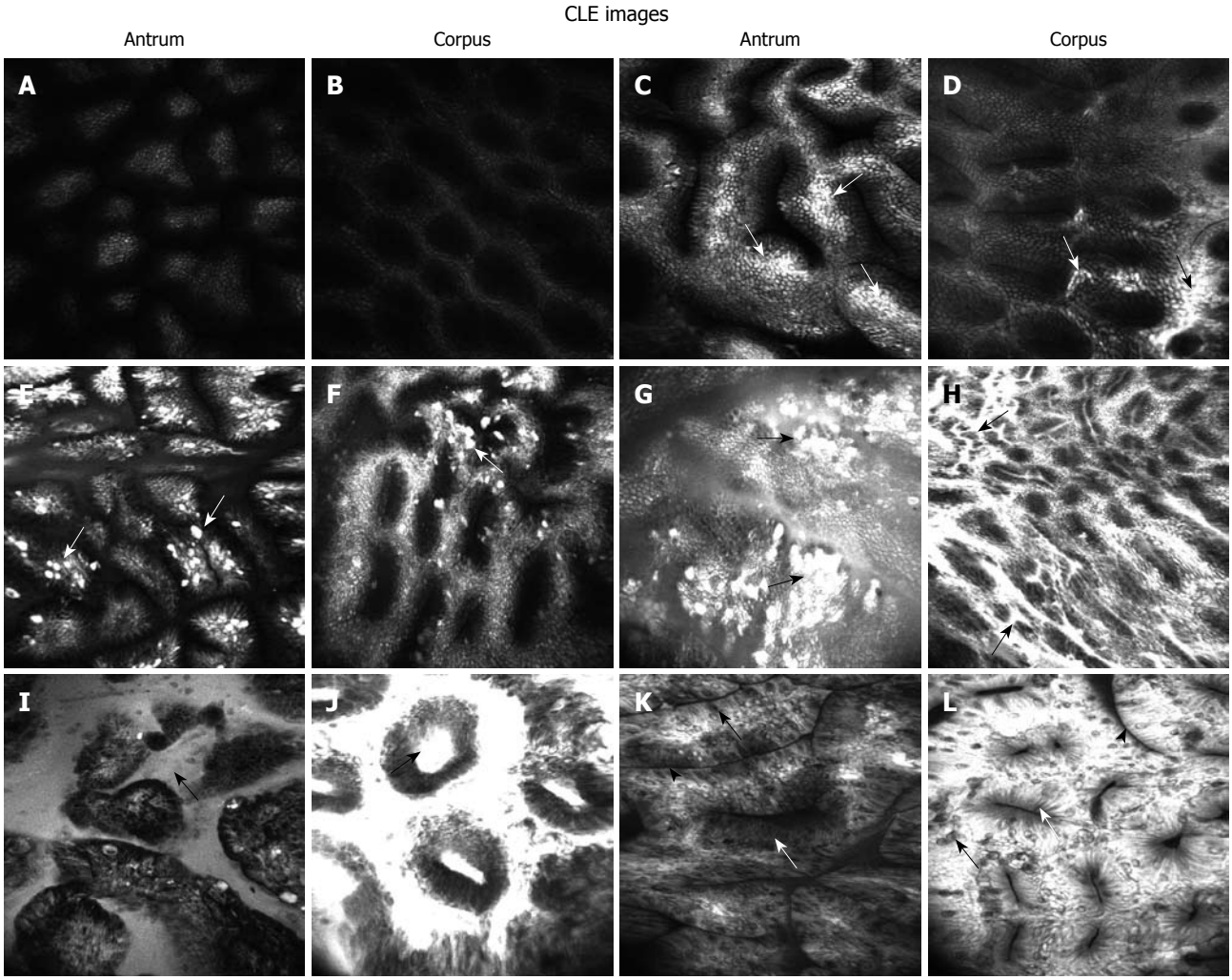

Figure 1 Confocal laser endomicroscopy classification of *Helicobacter pylori*-associated gastritis severity in gastric antrum and corpus. A, B: Normal mucosa with normal antral and corporal pits, and free of fluorescein leakage; C, D: Active inflammation (mild) with slightly distorted pits and intact epithelium, scattered focal fluorescein leakage (arrows); E, F: Active inflammation (moderate) with more distorted pits and partly destroyed epithelium (arrows), and more fluorescein leakage; G, H: Active inflammation (marked) with markedly distorted pits and dilated opening, destroyed epithelium (arrows), and widespread fluorescein leakage; I, J: Glandular atrophy with decreased gastric pits and markedly dilated opening (arrows); K, L: Intestinal metaplasia with villous-like gastric pits and goblet cells (black arrows), absorptive cells (white arrows) and brush border (arrowheads) appearing. CLE: Confocal laser endomicroscopy.

CLE criteria for glandular atrophy and IM: Glandular atrophy and IM are the severest stage of chronic gastritis. The CLE classification of glandular atrophy and IM was defined as decreased gastric pits with a dilated opening and as villous-like gastric pits with goblet cells, absorptive cells and brush border, respectively (Figure 1). To assess the CLE images, the mean number of gastric pits on each grade image was calculated and compared, except for that on IM images. The clinical values for IM features on CLE images were assessed by calculating their mean ratio present in each IM site on CLE images.

Final diagnosis of H. pylori infection

Rapid urease testing and Giemsa staining confirmed *H. pylori* infection. If only one test was positive for *H. pylori* infection, ^{13}C-urea breath test was performed for further confirmation. The *H. pylori* infection was classified into Hp (-), Hp (+), Hp (++) and Hp (+++).

Histopathology

All specimens were fixed in 10% formalin. An experienced histopathologist analyzed the histological features of each sample with hematoxylin and eosin staining and made the diagnosis according to the updated Sydney classification. The histological parameters assessed in this study included histological activity, chronic inflammation, glandular atrophy and intestinal metaplasia, which were identified blinding to the results of CLE or WLE.

Statistical analysis

Data were collected from CLE images and histological examination. analysis of variance and box-plot analysis of mCGS were used in pairwise comparison of mCGS among the 4 *H. pylori* test groups [Hp (-), Hp (+), Hp (++) and Hp (+++)]. $P < 0.05$ was considered statistically significant. The efficacy of CGS for predicting *H. pylori* infection was evaluated by area under the receiver oper-

Table 1 Characteristics of patients enrolled in this study

Patients	118
Gender	
Female	44
Male	74
Age (yr), median (range)	49.8 (19-67)
H. pylori infection (mCGS, mean ± SD)	
Positive	41
+	15 (1.11 ± 0.53)
++	15 (1.68 ± 0.51)
+++	11 (2.42 ± 0.45)
Negative	77 (0.61 ± 0.39)
Indication for CLE	
Upper GI symptoms	87
Screened for gastric cancer	31
Endoscopic diagnosis	
Normal stomach	6
Gastritis	92
Peptic ulcer	5
Polyps	13
Early gastric cancer	2

H. pylori: *Helicobacter pylori*; mCGS: Mean confocal gastritis score; CLE: Confocal laser endomicroscopy; GI: Gastrointestinal.

ating characteristic (ROC) curve analysis. On the other hand, the sensitivity, specificity, positive and negative predictive values (PPV, NPV) of the CLE criteria for diagnosing normal gastric mucosa, *H. pylori*-associated active inflammation, glandular atrophy and IM were calculated, respectively. Correlation between mCGS and mHGS was analyzed with the coefficient of determination. SPSS v16.0 (SPSS Inc., Chicago, IL) was used for all statistical analyses.

RESULTS

Collection of data

One hundred and eighteen patients including 74 males, at a mean age of 49.8 years (range 19-67 years) were enrolled in this study. A total of 12243 CLE images (mean 104 images per patient) and 653 optical biopsy images (mean 5.5 biopsy images per patient) were obtained. The median endoscopy time was 23.2 min (range 15-33 min) (Table 1). The quality of over 90% of CLE images was good, while that of the remaining 10% was not satisfactory because it was difficult to fix the distal tip on the gastric angle and the artifacts were motile in gastrointestinal tract. No endoscopic complications or adverse reactions to fluorescein were observed.

CLE diagnosis of H. pylori infection

Association between mCGS and *H. pylori* infection: Of the 118 patients enrolled in this study, 41 were positive for *H. pylori*. Detailed data on *H. pylori* level are shown in Table 1. The mCGS was significantly differed among Hp (-), Hp (+),Hp (++), and Hp (+++) groups ($P < 0.001$). The median mCGS was significantly higher in Hp (+) and Hp (+++) groups than in Hp (-) and Hp (++) groups ($P < 0.001$), and higher in Hp (++) group than in Hp (+) and Hp (-) groups ($P < 0.001$) (Figure 2).

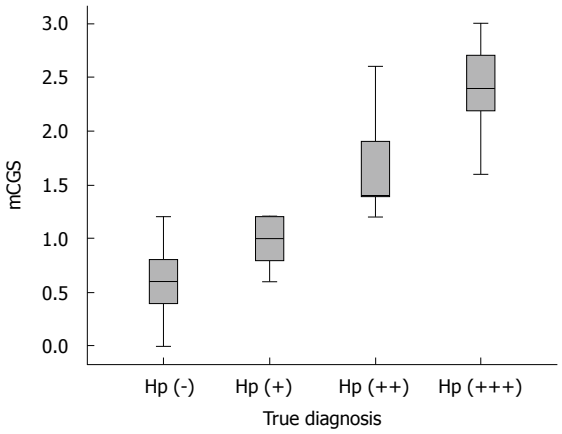

Figure 2 Box plot analysis of mean confocal gastritis score in 4 *Helicobacter pylori* test groups. mCGS: Mean confocal gastritis score.

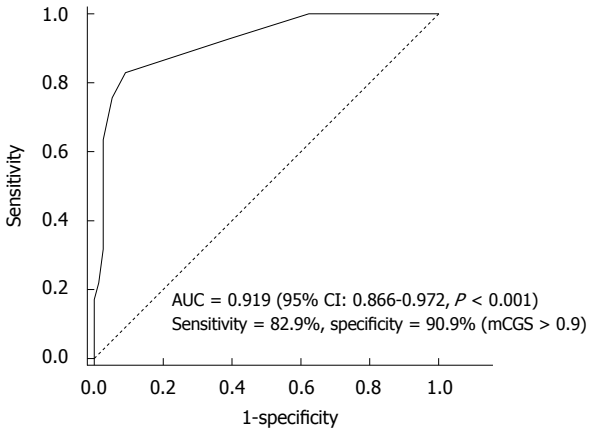

Figure 3 Receiver operating characteristic curve analysis showing the sensitivity and specificity of confocal laser endomicroscopy and mean confocal gastritis score for diagnosing *Helicobacter pylori* infection. AUC: Area under curve; mCGS: Mean confocal gastritis score.

Sensitivity and specificity of CLE for *H. pylori* detection: The ROC curve for mCGS was plotted to determine a cut-off value of sensitivity relative to specificity for diagnosing *H. pylori* infection. The area under the ROC curve was 0.919 (95% CI: 0.866-0.972), showing an excellent accuracy (Figure 3). The sensitivity and specificity of CEL were 82.9% and 90.9%, respectively, for mCGS > 0.9. With this cut-off value, CLE could correctly diagnose *H. pylori* infection.

CLE classification of histological gastritis severity

Normal gastric mucosa: A good correlation was found between normal mucosa detected by CLE and histology. The sensitivity, specificity, PPV and NPV of CLE were 94.6% (95% CI: 91.9%-97.4%), 97.4% (95% CI: 95.8%-99.1%), 96.5% (95% CI: 94.2%-98.7%), and 96.1% (95% CI: 94.1%-98.1%), respectively, for predicting gastric normal mucosa lesions (Table 2).

Chronic active inflammation: The CLE criteria correlated well with the active inflammation detected by histology. The sensitivity, specificity, PPV and NPV of CLE

Table 2 Sensitivity, specificity, positive predictive values and negative predictive values of confocal laser endomicroscopy for the diagnosis of normal mucosa, *Helicobacter pylori*-associated active inflammation, glandular atrophy and intestinal metaplasia (per specimen) [% (95% CI)]

CLE classification	Sensitivity	Specificity	PPV	NPV
Normal mucosa	94.6 (91.9-97.4)	97.4 (95.8-99.1)	96.5 (94.2-98.7)	96.1 (94.1-98.1)
Active inflammation	98.5 (97.2-99.8)	94.6 (91.9-97.4)	95.9 (93.7-97.9)	98.0 (96.3-99.7)
Glandular atrophy	92.9 (87.0-98.9)	95.2 (93.3-97.0)	72.5 (63.4-81.7)	98.9 (98.1-99.9)
Intestinal metaplasia	98.6 (95.7-100.0)	100 (100.0-100.0)	100 (100.0-100.0)	99.8 (99.4-100.0)

CLE: Confocal laser endomicroscopy; PPV: Positive predictive value; NPV: Negative predictive value.

Table 3 Confocal laser endomicroscopy and white-light endoscopy in predicting histological changes

Biopsy sites	CLE normal sites[1]	CLE abnormal sites[1]			
		Inflammation	Atrophy	IM	Early cancer
WLE normal sites	216	173	41	45	0
WLE abnormal sites[2]	31	83	25	23	2

[1]The normal and abnormal sites on confocal laser endomicroscopy (CLE) images were confirmed by histology; [2]Abnormal sites include erythema, erosion, nodules and petechiae, *etc.*, with no noticeable lesions such as ulcers or polyps on white light endoscopy (WLE) images. IM: Intestinal metaplasia.

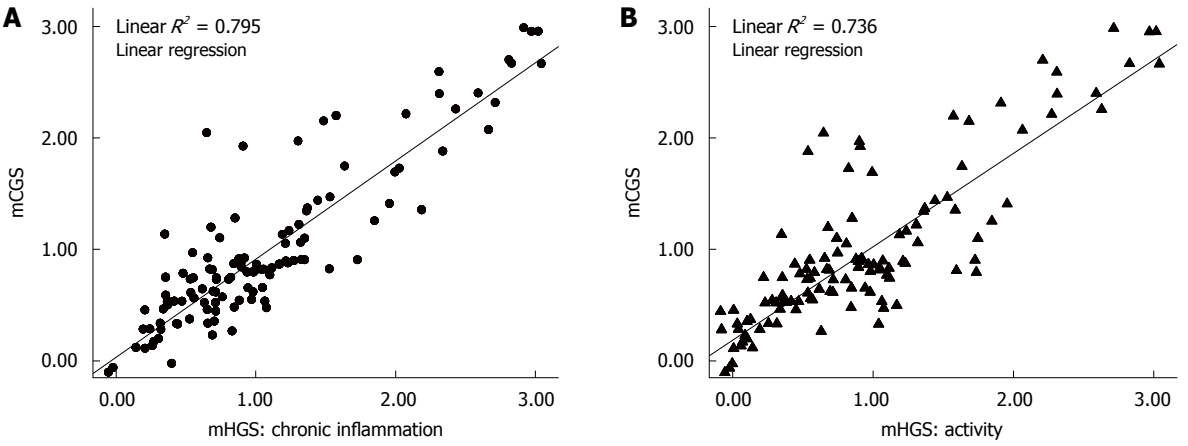

Figure 4 Correlation between mean confocal gastritis score and mean histologic gastritis score for histological chronic inflammation (R^2 = 0.795, A) and activity (R^2 = 0.736, B). mCGS: Mean confocal gastritis score; mHGS: Mean histologic gastritis score.

were 98.5% (95% CI: 97.2%-99.8%), 94.6% (95% CI: 91.9%-97.4%), 95.9% (95% CI: 93.7%-97.9%), and 98.0% (95% CI: 96.3%-99.7%), respectively, for predicting active inflammation detected by histology (Table 2). In particular, the histological activity and chronic inflammation were significantly differed among the 3 levels of active inflammation on CLE images, based on the evidence that histological activity and chronic inflammation were positively and linearly correlated with the mCGS (R^2 = 0.795, R^2 = 0.736, $P < 0.001$) (Figure 4).

Glandular atrophy and IM: The sensitivity, specificity, PPV and NPV of CLE were 92.9% (95% CI: 87.0%-98.9%), 95.2% (95% CI: 3.3%-97.0%), 72.5% (95% CI: 63.4%-81.7%) and 98.9% (95% CI: 98.1%-99.9%), respectively, for glandular atrophy (Table 2). Furthermore, a post-CLE analysis of CLE images showed that the mean number of gastric pits in glandular atrophy was significantly less than that in IM on CLE images ($P < 0.001$) (Figure 5).

The sensitivity, specificity, PPV and NPV of CLE for IM were 98.6% (95% CI: 95.7%-100.0%), 100% (95% CI: 100.0%-100.0%), 100% (95% CI: 100.0%-100.0%), and 99.8% (95% CI: 99.4%-100.0%), respectively, for predicting IM (Table 2). Of the IM features on CLE images, goblet cells and absorptive cells were more common and easier to note than the other features ($P < 0.001$) (Figure 6).

CLE and WLE for predicting histological conditions: In our study, 54.5% (259/475) normal sites were found to be abnormal on WLE by CLE (173 inflamed sites, 41 atrophic sites and 45 IM sites). In contrast, of the 164 abnormal sites on WLE images, 18.9% (31/164) were found to be normal on CLE images. All the findings on CLE images were confirmed by histology, indicating that CLE can show more histological lesions than WLE ($P < 0.001$) (Table 3).

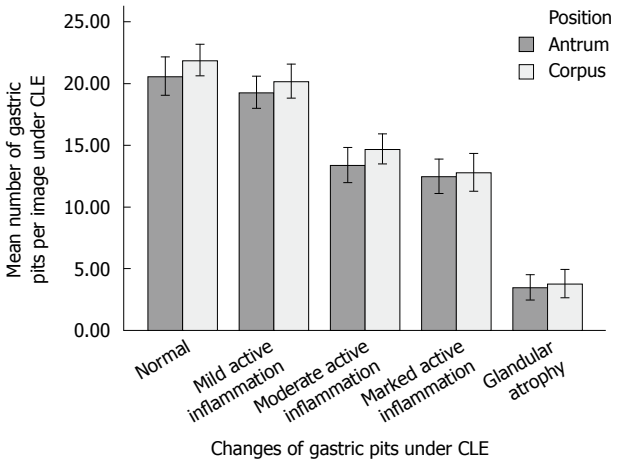

Figure 5 Gastric pits on confocal laser endomicroscopy images. CLE: Confocal laser endomicroscopy.

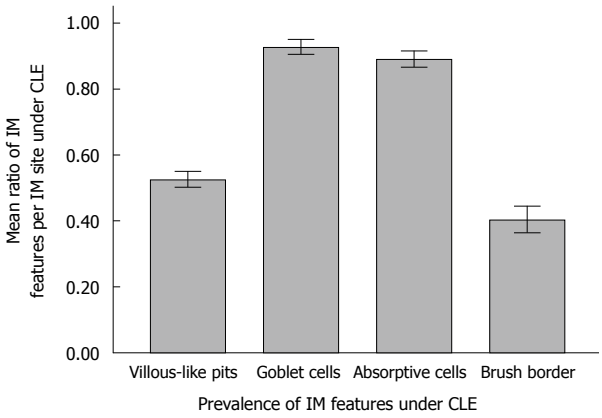

Figure 6 Prevalence of intestinal metaplasia features on confocal laser endomicroscopy images. IM: Intestinal metaplasia; CLE: Confocal laser endomicroscopy.

DISCUSSION

Conventional endoscopy is not frequently correlated with histological alterations, WLE is not accurate for predicting histological gastritis with nonspecific erythema, prominent area gastrica, nodularity, or erosions, *etc.*[16], and gastroscopy without biopsy is incomplete in routine clinical practice. In recent years, novel endoscopies, such as magnification endoscopy and narrow band imaging, have been evaluated for gastric pathologic conditions with a good accuracy[17]. High resolution, magnification and advanced electronic dye techniques, can identify the mucosal and vascular details of *H. pylori* infection and its related complications, with a high diagnostic sensitivity and specificity[18].

More details of gastric pits can be clearly observed on CLE images at the magnification × 1000. Zhang *et al*[14] reported that CLE can reveal inflamed pits with marked morphological alterations. The sensitivity and specificity of type B'+D' pits are 81.9% and 99.3%, respectively, for predicting moderate to severe active inflammation. In our study, *H. pylori* infection was positively correlated with progressively increasing changes in gastric pits due to direct injury caused by *H. pylori* and inflammatory infiltration. CLE could accurately distinguish normal mucosa from *H. pylori*-infected mucosa in the stomach. ROC curve analysis showed a high sensitivity (82.9%) and specificity (90.9%) of mCGS for diagnosing *H. pylori* infection. The mCGS cut-off value was 0.9, indicating an accurate and balanced result. Fluorescein leakage can classify active inflammation in ulcerative colitis[19] and fluorescein leakage into crypt lumen is positively correlated with histological active inflammation, because of increased colonic permeability. Fluorescein leakage is often present in the stomach, which contributes to the diagnosis of *H. pylori*-associated gastritis. When normal gastric mucosa is observed on CLE images, the fluorescence is confined to beneath the subepithelium and evenly distributed, epithelial cells are clearly visible and well-demarcated, showing a uniform gray cytoplasm and black border (Figure 1A and B). However, in *H. pylori*-infected mucosa, fluorescein easily leaks onto the surface through the damaged epithelium or normal epithelium with a high permeability. That is why this region is brighter than the surrounding normal epithelium. Sometimes, with a mass of the seepage, the entire field of vision can be white (Figure 1C-H). However, because fluorescein leakage is only an indirect response to mucosal damage and histological inflammation, the specificity of fluorescein is lower. In our study, fluorescein in combination with other CLE features was used to diagnose *H. pylori* infection following the active inflammation criteria defined by CLE[4]. The CLE scoring system used in our study could offer an objective evaluation of the entire stomach. High mCGS of the stomach indicated severe *H. pylori* infection, whereas low mCGS indicated mild or no *H. pylori* infection. Because the CGS agrees with the grading of confocal images, a high score represents severely injured gastric mucosa. It was reported that *H. pylori* organisms are usually observed singly or in groups along the surface epithelium but rarely in deeper mucosa[20]. Adherence of *H. pylori* to epithelial cells is the first step in *H. pylori* infection. When urease is secreted by *H. pylori,* and inflammatory and immune processes are activated by Vac A and Cag A toxin, the epithelium is depleted of mucin with irregular and missing cells ("drop-out cells"). In our study, similar alterations in moderate and severe active inflammation were found on CLE images, and clusters of destroyed epithelial cells (drop-out cells) on the two grade images were closely correlated with *H. pylori* infection, suggesting that CLE can display the drop-out cells because such microscopic lesions may progress to microerosions and precursors of gastric ulcer. However, when mCGS is less than 0.9, it can result in 7 false-positive and 7 false-negative diagnoses. False-positive cases are mainly diagnosed as mild active inflammation based on the CLE images of antrum, because normal mucosa on CLE images is sometimes misdiagnosed as mild inflammation when some fibrin or debris appears on the mucosal surface. The 7 false-negative cases are diagnosed as mild inflammation with a low *H. pylori* level and the corresponding mucosal changes are thus not obvious to be identified.

The second endpoint was to demonstrate the histologi-

cal severity of gastritis by CLE, which showed that the 6 CLE grades were well correlated with the corresponding parameters of histological gastritis. Active inflammation was classified into 3 levels by CLE. On the other hand, obvious drop-out cells were observed at the moderate and marked levels with a high risk for gastric ulcer formation and management of these groups of patients. The severity of chronic inflammation was much higher at moderate and marked levels on CLE images (R^2 = 0.736 and 0.795), probably because interstitial infiltration of inflammatory cells can easily squeeze the glands and damage the epithelium with an increasing severity of inflammation and *H. pylori* infection, leading to aggravated pit distortion and fluorescein leakage.

Gastric glands would be destroyed, and glandular atrophy and/or intestinal metaplasia would occur when they are infected with *H. pylori* and infiltrated with inflammatory cells. Glandular atrophy is manifested as thinner mucosa without gastric rugae but with visibly vascular pattern on WLE images[21]. However, gastric atrophy is poorly correlated with histological atrophy on WLE images[22]. It was reported that CLE shows a good sensitivity and specificity for gastric atrophy[14]. In our study, the diagnostic sensitivity and specificity of CLE were 92.9% and 95.2% for glandular atrophy. A further analysis of CLE-diagnosed glandular atrophy was performed with a more objective method. The mean number of gastric pits was calculated on different CLE images, and the mean number of pits in glandular atrophy was significantly less than that in gastritis on CLE images ($P < 0.001$). However, IM on CLE images was not analyzed because the gastric pits were replaced by intestinal mucosa, which became another form of atrophy or metaplasia. Although the sensitivity and specificity of CLE were high for glandular atrophy, the PPV was relatively low (72.5%) probably due to the low prevalence of atrophy in histology (71 sites/590 sites, 12.0%), indicating that there is a certain false positive rate when CLE is used to diagnose glandular atrophy, and identification of mild glandular atrophy is a big challenge for CLE.

Although the Sydney system has suggested a definition of endoscopic intestinal metaplasia for "grey-white patches with a slight opalescent tinge and/or a villous appearance on close inspection", studies demonstrated that these features are quite difficult to be identified by conventional endoscopy[21,23]. The detection of IM needs multiple biopsy specimens. Tahara *et al*[18] reported that magnifying narrow-band imaging endoscopy can show IM with a sensitivity of 73.3% and a specificity of 95.6%. In this study, IM was further analyzed following the CLE criteria[15]. The sensitivity and specificity of CLE were 98.6% and 100%, respectively, for IM in our study, and were higher than those reported by Tahara *et al*[18]. In addition, goblet cells and absorptive cells were more frequently and easily observed than other features or parameters in IM on CLE images in our study ($P < 0.001$). Because diagnosis of IM is often disregarded by naked eyes or by chromoendoscopy in clinical practice, more powerful endoscopic techniques, such as CLE and magnifying endoscopy, should be used to map IM[24].

Microscopic examination is essential for endoscopists to make a final diagnosis of gastritis, because the term of gastritis has been used indiscriminately and whether histological inflammation is present or not. However, CLE plays a pathological role in improving the diagnostic rate of WLE. In this study, CLE could discover 54.5% of lesions missed by WLE and correct 18.9% of normal mucosal sites misdiagnosed as abnormal sites by WLE, because the micro-gastric inflammation may appear completely normal on WLE images, suggesting that the presence or absence of gastritis should not be determined only based on gross WLE images. However, CLE can detect these micro-changes.

The main limitations of our study are as follows. First, we did not establish a control group, or a follow-up group to validate our hypothesis from multiple aspects, leading some bias in determination of *H. pylori* infection and histological gastritis. Second, we did not evaluate inter- and intra-observer variability. The repeatability of our CLE criteria should be assessed in a future single study. Third, due to the scanning depth and resolution limitations of CLE, there is still some gap when compared with *in vitro* histology. Therefore, the CLE equipment needs to be constantly upgraded.

In conclusion, *H. pylori* infection is related to its related CLE image features. Histologically active inflammation can be classified by CLE. Two most accurate markers, goblet cells and absorptive cells for IM, are established. Although CLE itself still has some limitations as a novel technique, it is a promising new procedure for accurate histological assessment *in vivo*.

COMMENTS

Background

The accurate diagnosis of chronic gastritis still relies on histopathology. Confocal laser endomicroscopy (CLE) can show gastric pit patterns and epithelial cells *in vivo*, thus, contributing to an accurate diagnosis of chronic gastritis *in vivo*.

Research frontiers

CLE has been extensively investigated in cancerous or precancerous mucosa of Barrett's esophagus and its related neoplasia, early esophageal cancer, gastric intestinal metaplasia, neoplasia and early cancer, colonic neoplasia and cancer, and ulcerative colitis, *etc*.

Innovations and breakthroughs

Endoscopic diagnosis of chronic gastritis and *Helicobacter pylori* (*H. pylori*) infection is still challenge to clinical endoscopists. This study first used CLE to classify *H. pylori* infection-associated chronic gastritis, which could accurately diagnose chronic gastritis *in vivo* without mutiple biopsy specimens.

Applications

By using the classification criteria for chronic gastritis established in this study, clinical endoscopits could use CLE to evaluate the conditions of the entire stomach, and calculate the abnormal mucosal sites in the entire stomach.

Terminology

CLE is novel digestive endoscope. It is a conventional white-light endoscope with a confocal laser microscope integrated into the distal tip. CLE can realize a histological-like examination during routine endoscopy and accurately diagnose gastrointestinal diseases at the magnification × 1000.

Peer review

The authors established the classification criteria for chronic gastritis, and compared CLE and white light endoscopy in diagnosis of gastritis and intestinal metaplasia, thus proving a novel method for the diagnosis of gastritis and intestinal metaplasia by CLE, which has a higher sensitivity and specificity than conventional endoscopy.

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BRIEF ARTICLE

Smad7 dependent expression signature highlights BMP2 and HK2 signaling in HSC transdifferentiation

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Abstract

AIM: To analyse the influence of Smad7, antagonist of transforming growth factor (TGF)-β canonical signaling pathways on hepatic stellate cell (HSC) transdifferentiation in detail.

METHODS: We systematically analysed genes regulated by TGF-β/Smad7 in activated HSCs by microarray analysis and validated the results using real time polymerase chain reaction and Western blotting analysis.

RESULTS: We identified 100 known and unknown targets underlying the regulation of Smad7 expression and delineated 8 gene ontology groups. Hk2, involved in glycolysis, was one of the most downregulated proteins, while BMP2, activator of the Smad1/5/8 pathway, was extremely upregulated by Smad7. However, BMP2 dependent Smad1 activation could be inhibited *in vitro* by Smad7 overexpression in HSCs.

CONCLUSION: We conclude (1) the existence of a tight crosstalk of TGF-β and BMP2 pathways in HSCs and (2) a Smad7 dependently decreased sugar metabolism ameliorates HSC activation probably by energy withdrawal.

Key words: Transforming growth factor-β; Smad7; Hepatic stellate cell; Gene regulation; Glucose metabolism; BMP2

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INTRODUCTION

Histopathological changes of chronic liver diseases usually

start with inflammatory hepatitis, followed by fibrosis and the final stage of cirrhosis, possibly leading to liver cancer. Hepatic fibrosis is characterized by increased and altered deposition of newly generated or deficiently degraded extracellular matrix (ECM) in response to injury[1]. Hepatic stellate cells (HSCs) are the major fibrotic precursor cells that transdifferentiate in inflammatory liver tissue to fibrogenic myofibroblasts (MFBs), by undergoing morphological changes, increased expression of α-SMA and synthesis of large amounts of ECM components[2].

Transdifferentiation of HSCs is driven by a variety of cytokines with transforming growth factor (TGF)-β playing a master role. It stimulates quiescent HSCs by paracrine and transdifferentiated MFBs by autocrine mechanisms activating intracellular Smad cascades. A great variety of cytokines, chemokines and mitogens (TNF-α, IFN-γ, EGF, PDGF, CTGF, ID1, YB1) display complex crosstalk with TGF-β[3-6].

Smad7 is a powerful antagonist of TGF-β in HSCs blunting downstream signaling by inhibiting receptor (R)-Smad phosphorylation[7]. In quiescent HSCs, expression of Smad7 itself is induced by the R-Smad cascade, thereby providing a negative feedback loop to terminate TGF-β signals[8]. We demonstrated before phenotypically and functionally that overexpressed Smad7 inhibits HSC transdifferentiation and attenuates the extent of fibrosis[7] suggesting that Smad7 is a promising antifibrotic tool for treatment approaches.

Therefore, in this study we analyzed the influence of Smad7 on the HSC gene expression pattern in great detail using microarray analysis. Its overexpression affects a great variety of cellular pathways involved in development, angiogenesis, differentiation, transcription, immune response, apoptosis, proliferation, signal transduction, ion and electron transport, sugar and lipid metabolism, morphogenesis, protein synthesis and modification, DNA synthesis and repair, cell adhesion, stress response, blood circulation, cell cycle and growth, cell motility, muscle contraction and organization of the cytoskeleton. The strongest regulated proteins are Pla2g2a, Cyp4b1, both upregulated and, Hk2 and VEGFa, which were downregulated significantly. Interestingly, BMP2, a member of the TGF-β family and alternative activator of the Smad1/5/8 pathway, was strongly induced by Smad7 overexpression in HSCs.

MATERIALS AND METHODS

Affymetrix gene chip array

Primary HSCs of male Sprague-Dawley were isolated as previously described[9,10]. To identify Smad7 dependent gene responses, HSCs were infected with adenoviruses encoding for Smad7 (AdSmad7; kindly provided by C. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden)) or LacZ (AdLacZ) as control 2 d after seeding[7].

RNA sample collection and generation of biotinylated complementary RNA probes was carried out according to the Affymetrix GeneChip® Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA,USA). In brief, total RNA was prepared at day 4 from 5×10^6 cultured primary HSCs that were infected with AdLacZ or AdSmad7 at day 2. Twenty five micrograms total RNA was reversely transcribed into double-stranded cDNA using HPLC-purified T7-(dt) 24 primers (MWG, Ebersberg, Germany) and the Superscript choice cDNA synthesis system (Invitrogen Corp., Carlsbad, CA). Purified cDNA was used to synthesize biotinylated complementary RNA using the BioArray High Yield RNA Transcription Labeling Kit (Enzo Diagnostics, Enzo Life Science Inc., Farmingdale, NY, USA). Each sample was hybridized to an Affymetrix rat Genome RG-U34A microarray (8799 probe sets) for 16h at 45℃. Expression values of each probe set were determined and AdSmad7 infected samples were compared to AdLacZ infected controls using the Affymetrix Microarray Suite 5.0 software.

Intensities across multiple arrays were normalized to a target intensity of 2500 using global normalization scaling. Two separate experiments with HSCs from different animals were performed under identical conditions. Genes whose expression levels were changed more than 2-fold with $P < 0.001$ in both experiments were considered to be significantly regulated by Smad7. These genes were investigated according to their molecular function and biological process by searching the gene ontology (GO) term database. Genes differentially expressed in AdSmad7 treated compared to controls were classified by "pathway" analysis [KEGG (http://www.genome.jp/kegg/pathway.html), PathwayArchitect, Stratagene].

Reverse-transcription and quantitative real-time polymerase chain reaction

Total RNA was collected from 3 (3 d-) or 7 d old (7 d-)HSCs, which were either infected with AdSmad7 or AdLacZ 2 d earlier or were uninfected[11]. cDNA from cell culture samples was synthesized as described[11]. Quantitative real-time polymerase chain reaction (RT-qPCR) was performed as in[11] with modified conditions: 95℃ for 60 s, then 40 cycles (50 cycles for low copy genes) of 95℃ for 10 s, 60℃ for 10 s and 72℃ for 15 s. Annealing temperature was set at 58℃ for U92564 and 62℃ for rat VEGF.1. Primers are listed in Table 1. The quantity of target mRNA was determined using a TGF-β RI standard curve[11]: A cDNA fragment was amplified and column-purified using the QIAquick PCR purification kit (Qiagen) and the following primers: TGFβ RI (GI: 416397) 180 bp; F (5'-CGTCTGCATTGCACTTATGC-3'), R (5'-AGCAGTGGTAAACCTGATCC-3'). A standard curve was generated from serial 10 time logarithmic dilutions of the cRNA by reverse transcription.

Western blotting analysis

Isolated primary HSCs of female Wistar rats were cultured as in[7]. Following overnight starvation (0.5% FCS) HSCs were stimulated with 5 ng/mL human recombinant TGF-β (Peprotech, Hamburg, Germany) or 20 ng/mL BMP2 (R&D, Minneapolis, MN), respectively.

Table 1 Primer used for quantitative real-time polymerase chain reaction validation of array results

Gene	Probe set ID	Forward	Reverse
CYP4B1	M29853	5'CCGAAGGCTGCAGATGTGT3'	5'TTTGGCCCATCCAGAACTAGTAG3'
mSmad7		5'GGTGCTCAAGAAACTCAAGG3'	5'CAGCCTGCAGTCCAGGCG3'
BMP2	L02678_at	5'TGCCCCCTAGTGCTTCTTAGAC3'	5'GGGAAGCAGCAACACTAGAAGAC3'
SGⅢ	U02983	5'CAAGCAGGACCGAGAATCAG3'	5'CGTTGGACAAGGTCAAGGTG3'
Zfp423	U92564	5'GCAGTGCTACACCTGACTCG3'	5'GTCATCCCGCATCTTCTTCTG3'
Pla2g2a	x51529	5'GCTCAATTCAGGTCCAGGG3'	5'CCACCCACACCACAATGG3'
EST189231	AA799734	5'CGGCTCACTGAGCTTGAAGTAG3'	5'ACACGACGGAGGAGCTTCTG3'
Olr1	AB005900	5'CAGAGAGAACTGAAGGAACAG3'	5'GGACCTGAAGAGTTTGCAGC3'
ID1	L23148_g_at	5'TGGACGAACAGCAGGTGAAC3'	5'TCTCCACCTTGCTCACTTTGC3'
HK2	D26393exon_s_at	5'CTCAGAGCGCCTCAAGACAAG3'	5'GATGGCACGAACCTGTAGCA3'
Slc16a3	U87627	5'CTCATCGGACCCCCATCAG3'	5'CGCCAGGATGAACACATACTTG3'
ratVEGF.1		5'TGCCAAGTGGTCCCAGGC3'	5'ATTGGACGGCAATAGCTGCG3'

For Smad7 overexpression studies, HSCs were infected on day 3 or day 6 with 50 IFU/cell (infectious units) Smad7 encoding adenovirus for 24 hr in medium containing 5% FBS. Four days old HSCs are considered to be in the transactivation process, while 7 d old HSCs are considered to be fully activated. After infection cells were serum-starved overnight and stimulated with 5 ng/mL TGF-β1 or 20 ng/mL BMP2. Generally, more than 90% of HSCs were infected.

For Western blotting analysis 20 μg protein was separated (4%-12% Bis-Tris Gel, NuPAGE, Invitrogen) and transferred to nitrocellulose membranes (Pierce, Rockford, IL). Nonspecific binding was blocked with 5% milk/TBST for Smad7 and GAPDH (Santa Cruz, CA, USA) or 5% BSA/TBST for pSmad1/3 antibodies (Epitomics/Biomol). Horseradish peroxidase-linked goat anti-rabbit antibody (Santa Cruz, CA, USA) served as secondary antibody. Membranes were developed with Supersignal Ultra (Pierce, Hamburg, Germany).

RESULTS

Smad7 dependent gene expression pattern

At day 2 of culture, primary rat HSCs were infected with AdSmad7 or AdLacZ (control). Two days later, when HSCs are in the process of transdifferentiation, the expression of genes displayed on 8799 probe sets was compared between cells overexpressing Smad7 and controls. Confirming Western blotting analysis of HSC lysates[12], microarray data revealed tremendous overexpression of Smad7 in AdSmad7 infected HSCs (40.79 times).

One hundred and twenty-nine probe sets were found differentially expressed due to Smad7, including 10 unknown proteins, 1 predicted protein and 89 known proteins (Table 2 provides a full list). According to their biological role, these genes were classified into eight main GO groups (Figure 1A). 37% of the regulated genes are involved in development. 22% can be assigned to signal transduction processes, which was expected since Smad7 represses TGF-β signaling and thus has impact on manifold different cross-talking signaling pathways. 15% refer to multicellular organismal processes (i.e. processes involved in intercellular interaction of any kind), 35% to response to stimulus, 21% to localization, 38% to metabolic processes, 25% to cell differentiation and 5% to cell adhesion. Note that the total percentage is greater than 100% as some regulated genes can be assigned to different ontology groups. A similar classification of all differentially expressed genes was carried out according to their molecular function (Figure 1B). Figures 2 and 3 graphically summarize regulation of all genes according to their ontology groups.

In general, many known mediators of TGF-β signaling were differentially expressed in AdSmad7 infected HSCs, confirming a direct link of Smad7 effects to TGF-β signalling (Table 2). ECM proteins like Col1a1 and Fn1 which are induced during HSC activation and fibrogenesis were negatively regulated upon Smad7 overexpression. Further profibrogenic cytokines like CXCL10 and HGF were upregulated. In addition, Cyp proteins like Cyp1b1, Cyp2E1 and Cyp4B1, Id proteins 1, 2, and 3, as well as PDGFR A were identified as Smad7 dependent in activated HSCs. Unexpectedly, several genes involved in glucose metabolism, so far annotated as predominantly associated with hepatocytes were influenced by Smad7 overexpression in HSCs.

As expected, Smad7 led to an opposite regulation of a number of recently systematically identified genes induced during HSC activation[14]. Table 3 contains a complete list of proteins identified to be regulated in both studies. In total, 37 genes of our study overlapped with the array results reported by[14]. Twenty-two of those (60%), e.g. HK2, were induced during activation[14] and decreased by Smad7 (this study) and therefore probably represent profibrogenic TGF-β target genes. There were also a few genes strongly upregulated by Smad7, which were downregulated during *in vivo* HSC activation, e.g. BMP2. Some of the proteins found to be differently regulated by activation *vs* Smad7 overexpression are already known to be TGF-β target genes and related to fibrogenesis, i.e. BMP2, Cnn1, Col1a1, Ddah1, Fn1, Lox, Pdgfra, Slc2a1, Slc16a3, and VEGF. Others might represent yet unidentified target genes of profibrogenic TGF-β signaling and/or new markers of HSC activation. Their specific influence on HSC transdifferentiation *in vivo* needs to be carefully investigated in future as they display potential antifibrotic target genes. In some cases De Minicis *et al*[14] reported opposite effects in regards to regulation of gene expression

Table 2 One hundred genes selected as being differentially expressed after Smad7 overexpression in hepatic stellate cells (note that some specific transcripts are detected by more than one probe set)

Official symbol	Average log2 fold	SD log2 fold	Affymetrix probe set ID	Official full name
Downregulated (n = 72)				
Acta2	-0.85	0.21	X06801cds_i_at	Smooth muscle α-actin
Ak3l1	-1.20	0.42	rc_AA891949_at	Adenylate kinase 3-like 1
Akap12	-1.00	0.57	U75404UTR#1_s_at	A kinase (PRKA) anchor protein (gravin) 12
Akr1b1	-0.70	0.42	M60322_g_at	Aldo-keto reductase family 1, member B1 (aldose reductase)
Atp6v1b2	-1.10	0.14	Y12635_at	ATPase, H transporting, lysosomal V1 subunit B2
Btg1	-0.65	0.49	L26268_g_at	B-cell translocation gene 1, anti-proliferative
Clec4f	-2.40	3.39	M55532_at	C-type lectin domain family 4, member f
Cml5	-1.30	0.42	rc_AA894273_at	Camello-like 5
Cnn1	-1.30	0.57	D14437_s_at	Calponin 1
Col1a1	-1.51	0.53	M27207mRNA_s_at/rc_AI231472_s_at/U75405UTR#1_f_at/Z78279_at/Z78279_g_at	Procollagen, type 1, α 1
Cryab	-1.13	0.32	M55534mRNA_s_at/X60351cds_s_at	Crystallin, α B
Cyp1b1	-1.10	0.28	rc_AI176856_at/U09540_at/U09540_g_at	Cytochrome P450, family 1, subfamily b, polypeptide 1
Ddah1	-0.95	0.21	D86041_at	Dimethylarginine dimethylaminohydrolase 1
Dpysl2	-0.95	0.64	rc_AA875444_at	Dihydropyrimidinase-like 2
Egr2	-1.25	0.64	U78102_at	Early growth response 2
Eif4ebp1	-1.05	0.07	U05014_at	Eukaryotic translation initiation factor 4E binding protein 1
Emp1	-0.65	0.78	Z54212_at	Epithelial membrane protein 1
Eno2	-0.80	0.71	X07729exon#5_s_at	Enolase 2, γ
Ercc1	-0.75	0.49	rc_AA892791_at	Excision repair cross-complementing rodent repair deficiency, complementation group 1
EST (unknown)	-2.65	0.64	rc_AI102814_at	EST
EST (unknown)	-2.60	0.28	rc_AI230256_at	EST
EST (unknown)	-2.00	0.14	rc_AA874889_g_at	EST
EST (unknown)	-1.40	0.85	rc_AA866419_at	EST
EST (unknown)	-1.35	0.64	X62950mRNA_f_at	EST
EST (unknown)	-1.10	0.99	rc_AA859740_at	EST
EST (unknown)	-0.85	0.35	rc_AA800708_at	EST
EST (unknown)	-0.40	1.41	X62951mRNA_s_at	EST
F3	-1.85	0.92	U07619_at	Coagulation factor Ⅲ
Fabp5	-0.80	0.57	S69874_s_at	Fatty acid binding protein 5, epidermal
Fkbp1a	-0.65	0.49	rc_AI228738_s_at	FK506 binding protein 1a
Fn1	-1.15	0.36	L00190cds#1_s_at/U82612cds_g_at/X05834_at	Fibronectin 1
Fntb	-1.28	0.49	rc_AI136396_at/rc_AI230914_at	Farnesyltransferase, CAAX box, β
Gabbr1	-1.25	0.92	rc_AI639395_at	γ-aminobutyric acid (GABA) B receptor 1
Gpx3	-1.10	0.14	D00680_at	Glutathione peroxidase 3
Hig1	-0.95	0.49	rc_AA891422_at	Hypoxia induced gene 1
Hk2	-3.20	0.00	D26393exon_s_at	Hexokinase 2
Id1	-2.55	0.35	L23148_g_at	Inhibitor of DNA binding 1
Id2	-2.45	0.21	rc_AI137583_at	Inhibitor of DNA binding 2
Id3	-1.85	0.13	AF000942_at/rc_AI009405_s_at	Inhibitor of DNA binding 3
Idi1	-0.70	0.57	AF003835_at	Isopentenyl-diphosphate delta isomerase
LOC686781	-1.25	0.21	rc_AA799657_at	Similar to NFκB interacting protein 1
Lox	-1.10	0.17	rc_AA875582_at/rc_AI234060_s_at/S77494_s_at	Lysyl oxidase
Lpl	-1.20	0.71	L03294_at/L03294_g_at/rc_AI237731_s_at	Lipoprotein lipase
Lrrc59	-0.65	0.64	D13623_at	Leucine rich repeat containing 59
Lum	-0.80	0.42	X84039_at	Lumican
Ncam1	-1.35	0.78	X06564_at	Neural cell adhesion molecule 1
Olr1	-2.43	0.99	AB005900_at/AB018104cds_s_at/rc_AI071531_s_at	Oxidized low density lipoprotein (lectin-like) receptor 1
P4ha1	-0.85	0.21	X78949_at	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), α 1 polypeptide
Pcsk6	-1.10	0.71	rc_AI230712_at	Proprotein convertase subtilisin/kexin type 6
Pfkp	-1.25	0.54	L25387_at/L25387_g_at	Phosphofructokinase, platelet
Plaur	-1.20	0.71	X71898_at	Plasminogen activator, urokinase receptor
Plod2	-1.00	0.28	rc_AA892897_at	Procollagen lysine, 2-oxoglutarate 5-dioxygenase 2
Pmepa1	-1.55	0.07	rc_AI639058_s_at	Prostate transmembrane protein, androgen induced 1
Ptk2	-0.85	0.35	S83358_s_at	PTK2 protein tyrosine kinase 2
Rasl11a	-2.25	0.49	rc_AI169372_at	RAS-like family 11 member A
Rasl11b	-1.03	0.24	rc_AA800853_at/rc_AA800853_g_at	RAS-like family 11 member B
Rcn2	-0.90	0.57	U15734_at	Reticulocalbin 2

RGD1306841	-1.10	0.14	rc_AI639203_at	Similar to RIKEN cDNA 2410006F12
RGD1310444_predicted	-1.25	0.21	rc_AA866432_at	LOC363015 (predicted)
Rgs4	-1.45	0.64	U27767_at	Regulator of G-protein signaling 4
Sc4mol	-1.10	0.45	E12625cds_at/rc_AI172293_at	Sterol-C4-methyl oxidase-like
Schip1	-1.00	0.42	rc_AA800036_at	Schwannomin interacting protein 1
Serpine1	-1.90	0.00	M24067_at	Serine (or cysteine) peptidase inhibitor, clade E, member 1
Slc12a2	-0.80	0.99	AF051561_s_at	Solute carrier family 12, member 2
Slc16a3	-2.05	0.35	U87627_at	Solute carrier family 16 (monocarboxylic acid transporters), member 3
Slc2a1	-1.15	0.35	S68135_s_at	Solute carrier family 2 (facilitated glucose transporter), member 1
Spink8	-2.55	1.48	rc_AA799734_at	Serine peptidase inhibitor, kazal type 8
Tfrc	-0.90	0.42	M58040_at	Transferrin receptor
Tnc	-0.90	0.28	U09401_s_at	Tenascin C
Tnnt2	-1.70	0.42	M80829_at	Troponin T2, cardiac
Vegfa	-2.25	1.28	L20913_s_at/M32167_g_at/rc_AA850734_at	Vascular endothelial growth factor A
Wfdc1	-1.70	0.14	AF037272_at	WAP four-disulfide core domain 1
Up-regulated (n = 28)				
Adora2a	0.85	0.35	S47609_s_at	Adenosine A2a receptor
Agtr1a	0.95	0.21	M74054_s_at/X62295cds_s_at	Angiotensin Ⅱ receptor, type 1 (AT1A)
Bmp2	2.83	1.31	L02678_at/rc_AA997410_s_at	Bone morphogenetic protein 2
Col3a1	0.90	0.42	M21354_s_at/X70369_s_at/	Procollagen, type Ⅲ, α 1
Cxcl10	1.05	0.21	U17035_s_at	Chemokine (C-X-C motif) ligand 10
Cyp2e1	1.00	0.14	M20131cds_s_at	Cytochrome P450, family 2, subfamily e, polypeptide 1
Cyp4b1	3.25	0.49	M29853_at	Cytochrome P450, family 4, subfamily b, polypeptide 1
Ednrb	0.70	0.42	rc_AA818970_s_at	Endothelin receptor type B
Ephx1	1.15	0.21	M26125_at	Epoxide hydrolase 1, microsomal
EST (unknown)	0.80	0.28	rc_AA874873_g_at	EST
EST (unknown)	0.90	0.28	rc_AI177256_at	EST
Glul	0.90	0.23	M91652complete_seq_at/rc_AA852004_s_at	Glutamate-ammonia ligase (glutamine synthase)
Hgf	1.03	0.05	E03190cds_s_at/X54400_r_at	Hepatocyte growth factor
Hsd11b1	0.95	0.49	rc_AI105448_at	Hydroxysteroid 11-β dehydrogenase 1
Igfbp3	1.15	0.30	M31837_at	Insulin-like growth factor binding protein 3
Kif4	1.05	0.07	rc_AA859926_at	Kinesin family member 4
Lhx2	0.95	0.21	L06804_at	LIM homeobox protein 2
Notch1	0.80	0.42	X57405_g_at	Notch gene homolog 1 (Drosophila)
Nr2f1	0.95	0.21	U10995_g_at	Nuclear receptor subfamily 2, group F, member 1
Pdcd4	1.00	0.14	rc_AI172247_at	Programmed cell death 4
Pdgfra	1.10	0.28	rc_AI232379_at	Platelet derived growth factor receptor, α polypeptide
Pla2g2a	3.60	0.00	X51529_at	Phospholipase A2, group ⅡA (platelets, synovial fluid)
Ptn	2.10	0.57	rc_AI102795_at	Pleiotrophin
Scg3	2.70	0.85	U02983	Secretogranin Ⅲ
Serping1	0.85	0.21	rc_AA800318_at	Serine (or cysteine) peptidase inhibitor, clade G, member 1
Smad7	5.35	1.20	AF042499_at	MAD homolog 7 (Drosophila)
Sod3	1.05	0.07	Z24721_at	superoxide dismutase 3, extracellular
Zfp423	1.85	1.47	U92564_at/U92564_g_at	Zinc finger protein 423

The average change in expression after Smad7 overexpression is given as log2 fold. SD: Square root of the variance; NFκB: Nuclear factor κB.

in "culture activated" cells compared to "*in vivo* activated" cells. This leaves a final evaluation of Smad7 influence on the regulation of these genes in HSC activation processes open.

Confirmation of array data using quantitative real-time PCR

To validate our microarray results, we selected 12 genes from array data identified as highly regulated in dependency to Smad7 for RT-qPCR analysis. Transdifferentiating (3 d in culture) and fully activated (7 d in culture) HSCs were investigated. TGF-β RI mRNA expression is not modulated during transdifferentiation[15,16] and was used as the expression reference. A synopsis of Smad7 associated modulation of gene expression, given in Figure 4 as log2 fold of LacZ, generally supports the array results. We confirmed upregulation of Cyp4B1, BMP2, SGIII, Zfp423, Pla2g2a and downregulation of EST189231, Olr1 and Id1 (Table 4) independent of time during the transdifferentiation process.

Interestingly, when comparing 3 d- with 7 d-HSCs, opposite effects of Smad7 were found for HK2 (0.38-fold in 3 d-, 3.85-fold in 7 d-HSCs), Slc16a3 (0.59-fold in 3 d, 2.25-fold in 7 d-HSCs) and VEGF.1 (0.51-fold in 3 d-, 1.07-fold in 7 d-HSCs), underlining temporal differences and modulation of the TGF-β signal during HSC activation[15].

Smad7 inhibits BMP2 dependent Smad1 expression

BMP2 was strongly upregulated in Smad7 expressing HSCs (Table 2, Figure 4A and B). Here, we further demonstrate that nevertheless Smad7 blunted BMP2 and

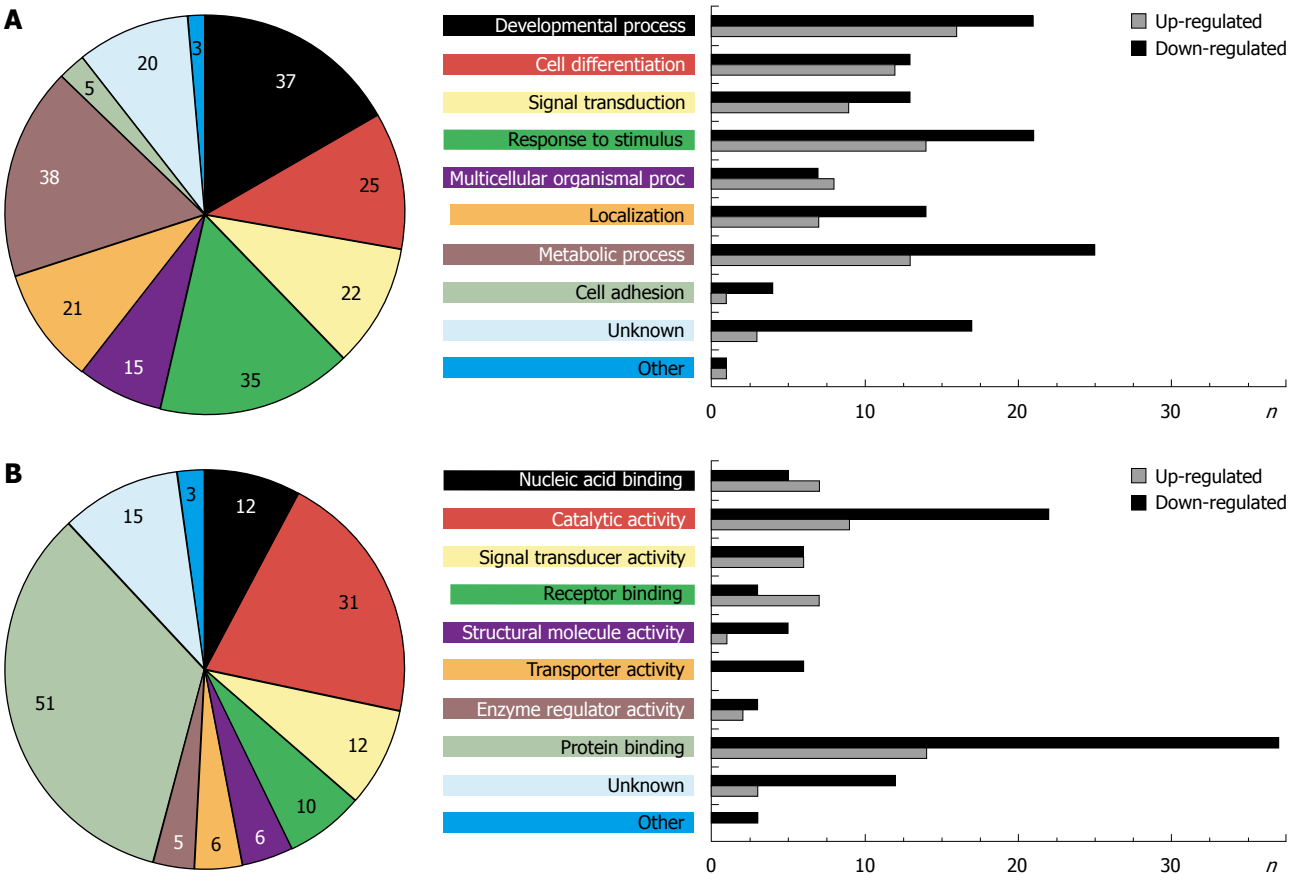


Figure 1 Genes whose expression levels were changed (*n* = 100) after overexpression of Smad7 in hepatic stellate cells are matched to 8 gene ontology annotations using GoMiner[13]. Left part: Percentage of genes matching to the given gene ontology. Note the total percentage is greater than 100% as the same regulated genes can be assigned to different annotations; Right part: Bar diagram shows number of upregulated (grey bars) and downregulated (black bars) genes matching to the given gene ontology (unknown = percentage/number of genes without annotation, other = percentage/number of genes which are not assignable to the given annotations). A: GO group: biological process; B: GO group: molecular function.

TGF-β dependent signalling *via* Smad1 upon AdSmad7 infection in transdifferentiating (4 d in culture) rat HSCs (Figure 5) and infected CFSC (data not shown). Fully activated (7 d in culture) HSCs, which are insensitive to TGF-β, remain responsive to BMP2 mediated Smad1 phosphorylation, show the same tendency when stimulated with BMP2.

DISCUSSION

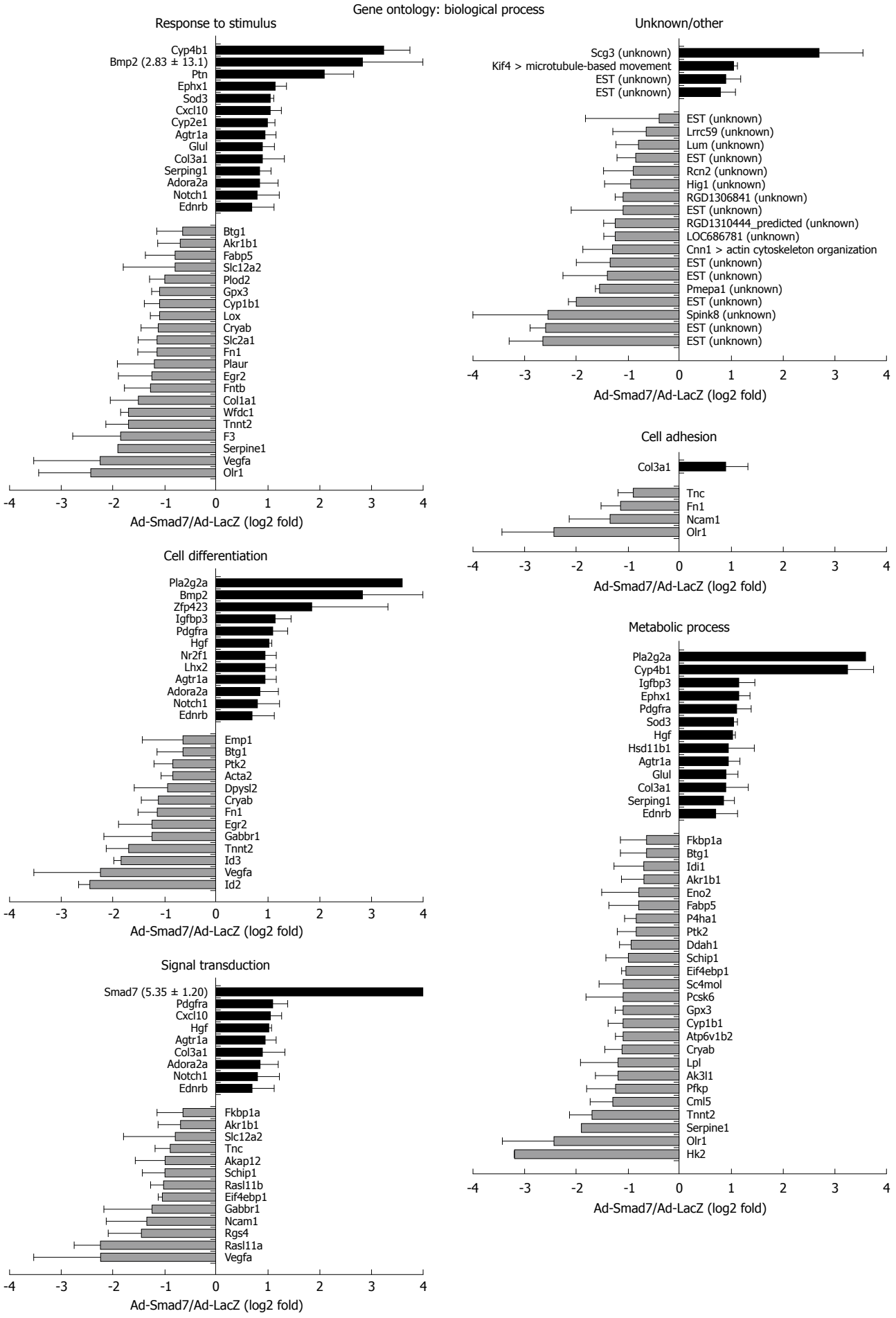
Using the Affymetrix Microarray approach, we systematically analyzed the effects of Smad7 overexpression during HSC transdifferentaition. About 100 genes were identified to be regulated upon Smad7 overexpression. For obvious reasons, only some of the regulated genes can be discussed below in detail. Nevertheless, all gene expression changes found constitute potential starting points for future research projects to unravel the process of liver fibrogenesis.

Tumor supressor genes were upregulated by Smad7 overexpression in HSCs

Pla2g2a and Cyp4B1 were strongly upregulated after Smad7 overexpression in HSCs. Pla2g2a participates in lipid metabolism/catabolism and was previously described as a tumor repressor in different cancer models, i.e. intestinal tumorigenesis, neuroblastoma, melanoma and colon cancer cell lines[17,18]. VEGF and Glut1, both known to be upregulated in tumor cells[19,20] were Smad7 dependently downregulated in HSCs. These results suggest an influence of Smad7 on tumor development and progression which is a long debated issue considering its regulative impact on ambiguous TGF-β signaling in tumorigenesis.

Cytochrome P450s are haem-thiolate proteins involved in oxidative degradation of particularly environmental toxins and mutagens and play a role in electron transport reactions. Additionally, they are key players in alcohol induced oxidative stress in liver, causing hepatocyte necrosis, apoptosis and liver fibrosis[21]. During steatosis, lipid peroxidation by Cyp2E1 is associated with inflammation and HSC activation including increased TGF-β production, possibly through up-regulation of KLF6[22]. Members of the Cyp P450 family are also upregulated during HSC activation[14].

Interestingly, overexpression of Smad7 increased the expression of some members of the Cytochrome P450 system through HSC activation, i.e. Cyp4B1 which is important in the metabolism of drugs, cholesterol, steroids and lipids, and Cyp2E1, while others are downregulated


Gene ontology: biological process
Response to stimulus
Cyp4b1
Bmp2 (2.83 ± 13.1)
Ptn
Ephx1
Sod3
Cxcl10
Cyp2e1
Agtr1a
Glul
Col3a1
Serping1
Adora2a
Notch1
Ednrb
Btg1
Akr1b1
Fabp5
Slc12a2
Plod2
Gpx3
Cyp1b1
Lox
Cryab
Slc2a1
Fn1
Plaur
Egr2
Fntb
Col1a1
Wfdc1
Tnnt2
F3
Serpine1
Vegfa
Olr1
-4 -3 -2 -1 0 1 2 3 4
Ad-Smad7/Ad-LacZ (log2 fold)
Unknown/other
Scg3 (unknown)
Kif4 > microtubule-based movement
EST (unknown)
EST (unknown)
EST (unknown)
Lrrc59 (unknown)
Lum (unknown)
EST (unknown)
Rcn2 (unknown)
Hig1 (unknown)
RGD1306841 (unknown)
EST (unknown)
RGD1310444_predicted (unknown)
LOC686781 (unknown)
Cnn1 > actin cytoskeleton organization
EST (unknown)
EST (unknown)
Pmepa1 (unknown)
EST (unknown)
Spink8 (unknown)
EST (unknown)
EST (unknown)
-4 -3 -2 -1 0 1 2 3 4
Ad-Smad7/Ad-LacZ (log2 fold)
Cell adhesion
Col3a1
Tnc
Fn1
Ncam1
Olr1
-4 -3 -2 -1 0 1 2 3 4
Ad-Smad7/Ad-LacZ (log2 fold)
Cell differentiation
Pla2g2a
Bmp2
Zfp423
Igfbp3
Pdgfra
Hgf
Nr2f1
Lhx2
Agtr1a
Adora2a
Notch1
Ednrb
Emp1
Btg1
Ptk2
Acta2
Dpysl2
Cryab
Fn1
Egr2
Gabbr1
Tnnt2
Id3
Vegfa
Id2
-4 -3 -2 -1 0 1 2 3 4
Ad-Smad7/Ad-LacZ (log2 fold)
Metabolic process
Pla2g2a
Cyp4b1
Igfbp3
Ephx1
Pdgfra
Sod3
Hgf
Hsd11b1
Agtr1a
Glul
Col3a1
Serping1
Ednrb
Fkbp1a
Btg1
Idi1
Akr1b1
Eno2
Fabp5
P4ha1
Ptk2
Ddah1
Schip1
Eif4ebp1
Sc4mol
Pcsk6
Gpx3
Cyp1b1
Atp6v1b2
Cryab
Lpl
Ak3l1
Pfkp
Cml5
Tnnt2
Serpine1
Olr1
Hk2
-4 -3 -2 -1 0 1 2 3 4
Ad-Smad7/Ad-LacZ (log2 fold)
Signal transduction
Smad7 (5.35 ± 1.20)
Pdgfra
Cxcl10
Hgf
Agtr1a
Col3a1
Adora2a
Notch1
Ednrb
Fkbp1a
Akr1b1
Slc12a2
Tnc
Akap12
Schip1
Rasl11b
Eif4ebp1
Gabbr1
Ncam1
Rgs4
Rasl11a
Vegfa
-4 -3 -2 -1 0 1 2 3 4
Ad-Smad7/Ad-LacZ (log2 fold)

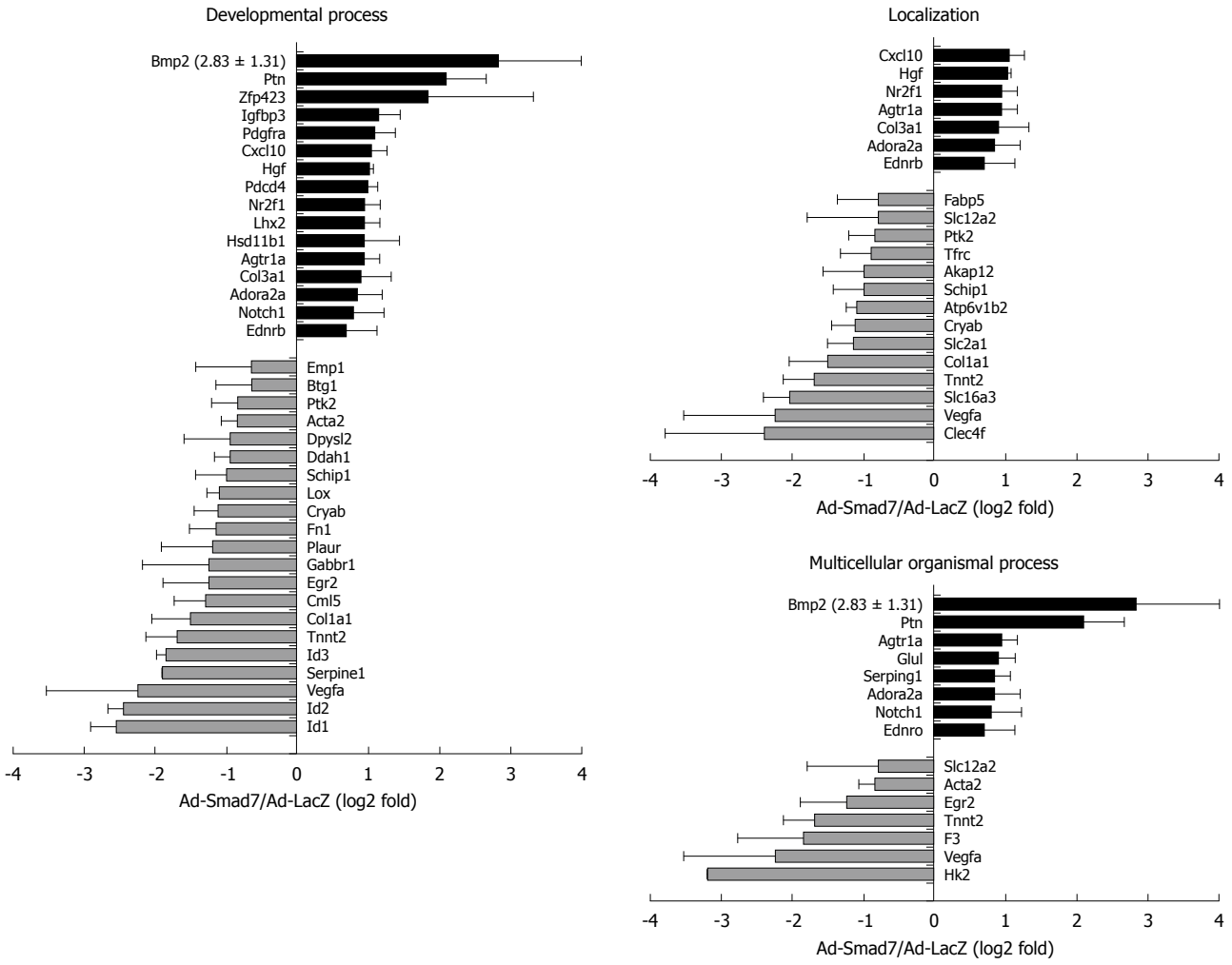
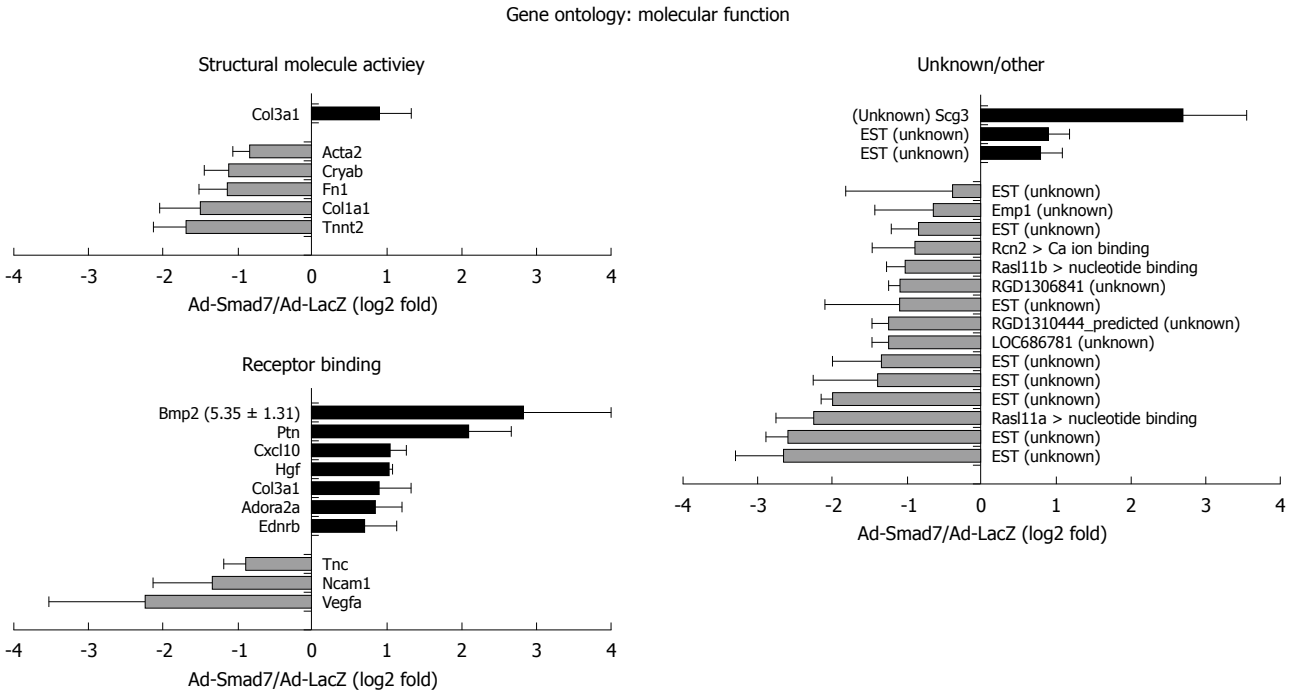


Figure 2 Genes with changed expression levels after overexpression of Smad7 are matched to 8 gene ontology terms of the biological process and to unknown/other. Unknown: Genes without annotation; Other: Genes with another annotation not assignable to the given annotations, details in brackets. Change of expression is given as log2 value of the fold factor with the SD. Black bars: Upregulated; Gray bars: Downregulated.



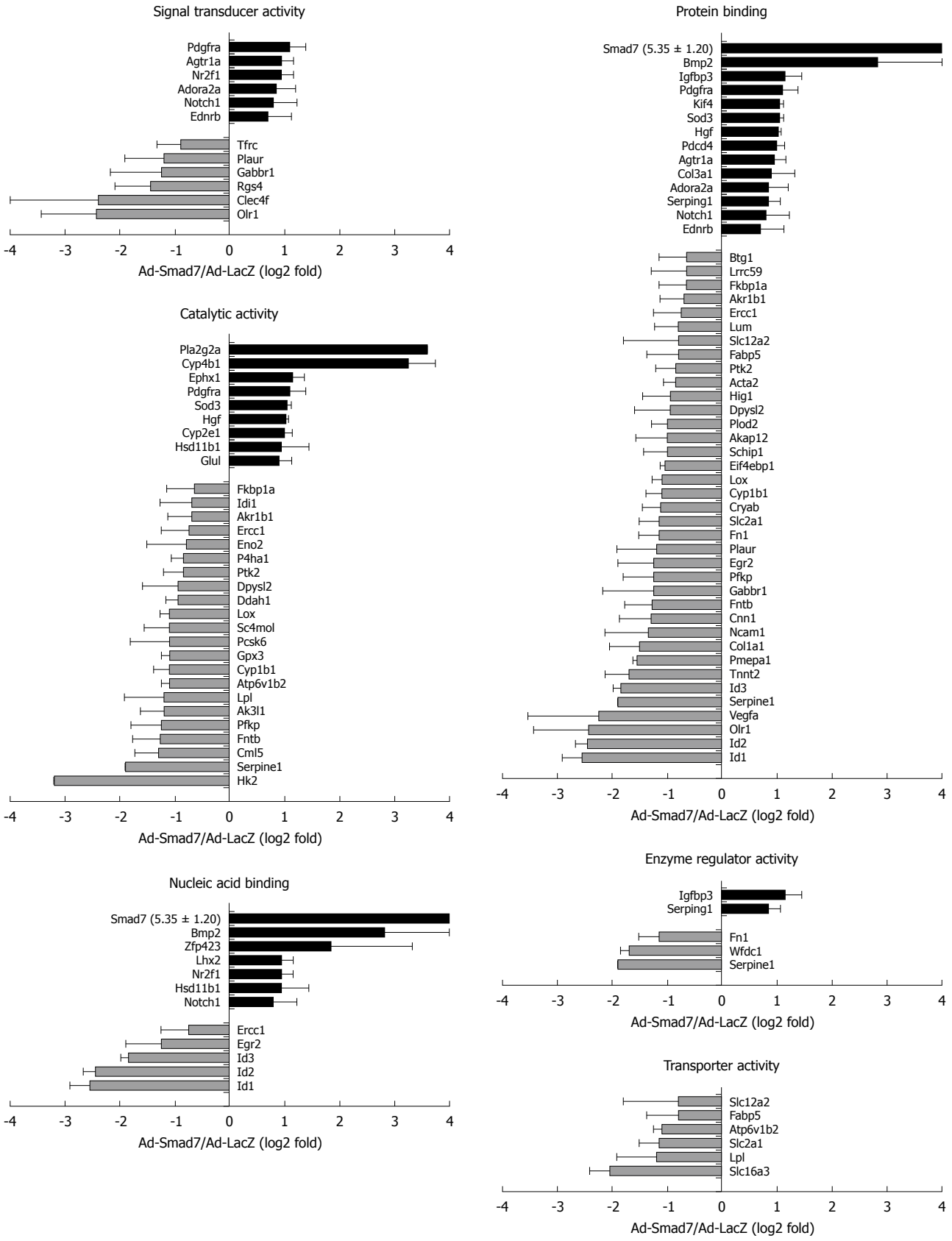


Figure 3 Genes whose expression levels were changed after overexpression of Smad7 are matched to 8 gene ontology terms of the molecular function and to unknown/other. Unknown: Genes without annotation; Other: Genes with another annotation not assignable to the given annotations, details in brackets. Change of expression is given as log2 value of the fold factor with the SD. The term nucleic acid binding includes the annotations nucleic acid binding, transcription factor activity, RNA binding, and transcription regulator activity. Black bars: Upregulated; Gray bars: Downregulated.

Table 3 Comparison of gene regulation in activated hepatic stellate cells *in vivo* (De Minicis *et al*[113])

Gene symbol	Smad7 overexpressing HSCs	*In vivo* activated untransformed HSCs
Acta2	↓	↑
BMP2[1]	↑	↓
Cnn1	↓	↑
Col1a1	↓	↑
Col3a1	↑	↑
Cryab	↓	↓
Cyp1b1	↓	↑
Ddah1	↓	↑
Ednrb	↑	↑[2]
Eno2	↓	↓
Ephx1	↑	↑[2]
Fn1	↓	↑
Gabbr1	↓	↓
Hgf	↑	↑[2]
Hk2[1]	↓	↑
Hsd11b1	↑	↑[2]
Id1	↓	↓
Igfbp3	↑	↑[2]
Kif4	↑	↑
Lox	↓	↑
Lpl	↓	↑[2]
Lum	↓	↑
Ncam1	↓	↑
P4ha1	↓	↑
Pdgfra	↑	↓
Pfkp	↓	↑
Plod2	↓	↑
Rasl11b	↓	↑
Serping1	↑	↑
Slc16a3	↓	↑
Slc2a1	↓	↑
Sod3	↑	↑
Tmepai_predicted	↓	↓[2]
Tnc	↓	↓[2]
Tnnt2	↓	↑
VEGFa[1]	↓	↑ (VEGFc)[1]
Wfdc1	↓	↑[2]

Gene expression profiles during hepatic stellate cell activation in culture and *in vivo* (*Gastroenterology* 2007; 132: 1937-1946[113]) and Smad7 overexpressing hepatic stellate cells (HSCs) (our study). [1]Genes regulated the strongest in our study are marked; [2]The regulation of *in vivo* activated HSCs which is different compared to culture activated HSCs in De Minicis study. In total 37 genes overlap in both studies, 22 of those (60%) are oppositely regulated indicating participation of transforming growth factor-β/Smad7 on the regulation of those genes *in vivo*.

upon Smad7 overexpression, e.g. Cyp1B1. These ambiguous effects probably reflect the complex control of oxidative metabolism in the cell.

Glucose metabolism and angiogenesis/vascularisation is downregulated by Smad7

Hk2 is a hexokinase, one of the best known enzymes of glycolysis, and is involved in cell cycle progression. According to the results of the microarray analysis it represents the most downregulated gene in AdSmad7 infected HSCs. One feature of activated HSCs is the ability to proliferate. TGF-β antagonizes proliferation in quiescent HSCs, whereas it has a growth promoting effect in transdifferentiated MFBs. Thus, Hk2 might be induced by TGF-β in HSCs during activation, subsequently stimulating HSC

Table 4 Comparison of gene regulation according to quantitative real-time polymerase chain reaction analysis and array analysis

Gene	Probe set ID	Array	RT-PCR analysis
Cyp4B1	M29853_at	Up	Up
Smad7	AF042499_at	Up	Up
BMP2	L02678_at/ rc_AA997410_s_at	Up	Up
SGIII	U02983_at	Up	Up
Zfp423	U92564_at/ U92564_g_at	Up	Up
Pla2g2a	x51529_at	Up	Up
EST189231	AA799734_at	Down	Down
Olr1	AB005900_at/ AB018104cds_s_at/ rc_AI071531_s_at	Down	Down
ID1	L23148_g_at	Down	Down
HK2	D26393exon_s_at	Down	3 d down/**7 d up**
Slc16a3	U87627_at	Down	3 d down/**7 d up**
VEGFa/ratVEGF.1 in RT-PCR	L20913_s_at/ M32167_g_at	Down	3 d down/**7 d up**

Different results in array and quantitative real-time polymerase chain reaction (RT-PCR) analysis are marked bold.

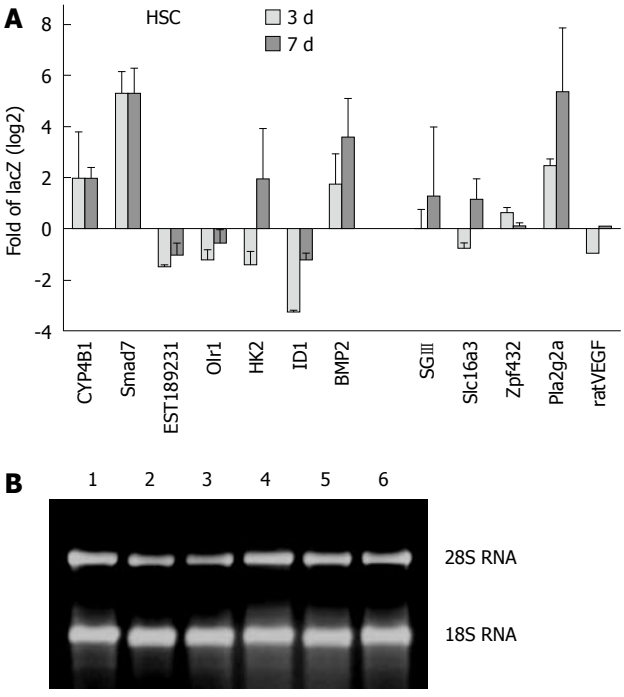


Figure 4 Validation of microarray results using quantitative real-time polymerase chain reaction. A: SYBR Green I-based real-time quantification to compare the mRNA expression patterns of 12 selected genes in hepatic stellate cell which were infected either with AdLacZ or AdSmad7. Transforming growth factor-β RI, not affected by Smad7 overexpression, served as a house-keeping gene. Results are given as relative expression of log2 fold of LacZ. 3 d (light grey column) and 7 d (dark grey column): 3 d and 7 d after adenoviral infection. Values are the mean of three measurements each performed in duplicates ± SD from independent experiments; B: Total RNA purity and integrity was verified by formaldehyde agarose gel electrophoresis. Lane 1: LacZ, 7 d; Lane 2: LacZ, 3 d; Lane 3: Smad7, 7 d; Lane 4: Smad 7, 3 d; Lane 5: Untreated control, 7 d; Lane 6: Untreated control, 3 d.

proliferation and thus providing at least part of the growth stimulatory effect of TGF-β. Although physiological ef-

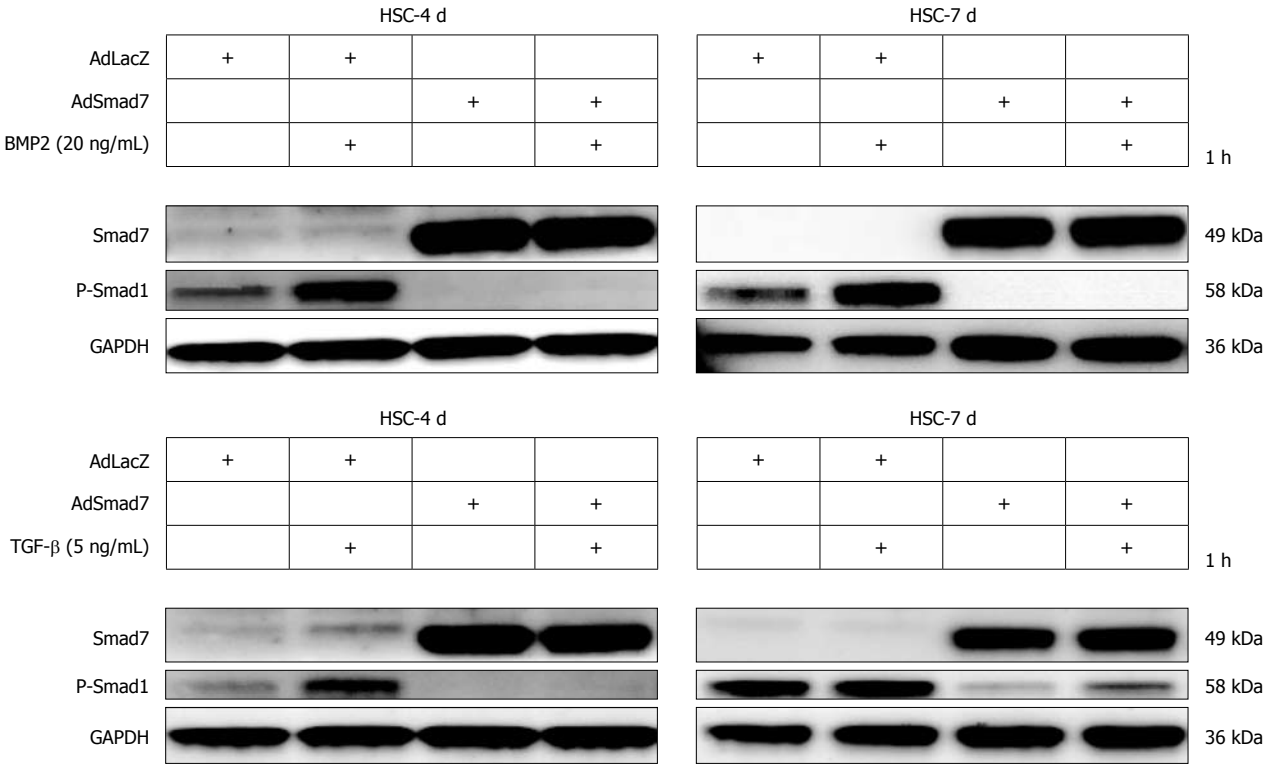


Figure 5 Smad7 overexpression inhibits BMP2 and transforming growth factor-β dependent Smad1 phosphorylation. Transdifferentiating (4 d old) and fully activated (7 d old) hepatic stellate cells (HSCs) infected or non-infected with either AdLacZ (control) or AdSmad7 were stimulated for 1 h with 20 ng/mL BMP2 or 5 ng/mL transforming growth factor (TGF)-β as indicated. Smad7 overexpression and Smad1 phosphorylation were analysed using Western blottings. GADPH served as a reference. While transdifferentiating HSCs are sensitive to both BMP2 and TGF-β stimulation, fully activated HSCs are only responsive to BMP2.

fects of glucose metabolism in the liver are traditionally associated to hepatocytes and provide a direct link to fibrogenesis *via* hyperglycemia and insulin resistance[23-25], one could speculate that activated HSCs need more energy and thus, glycolysis is upregulated TGF-β dependently in this cell type. In line, HSCs become sensitive to glucose signaling during activation, high glucose concentrations stimulate ROS production through PKC-dependent activation of NADPH oxidase and induce MAP kinase phosphorylation subsequent to proliferation and type I collagen production in this cell type[26] suggesting a crucial role of HSC-sugar metabolism in fibrogenesis.

Upregulation of Hk2 during activation of HSCs further suggests that glycolysis induction and increased levels of involved proteins may occur by other means than elevated blood glucose levels ([14], our study). This in turn indicates a direct connection between fibrosis and enhanced glycolysis independent of inducing external stimuli of either process.

Beside Hk2 regulation, other genes encoding enzymes of glycolysis (Eno2, PFKP) or related to glucose metabolism were downregulated Smad7 dependently, e.g. VEGFa, PAI-1 (Serpine1), F3, Slc2a1 (Glut1), FN1, EIF4ebp1 and PTK2 (Figure 6). A list of all references proving the relation of these genes to glucose metabolism will be provided to interested readers on request.

Glut1 is a glucose transporter protein which becomes upregulated in activated HSCs or upon HSC activation[14]. Since Smad7 decreases Glut1 expression levels and other proteins involved in glycolysis, TGF-β seems to enhance glucose metabolism and energy supply during HSC activation thus enabling the cells to proliferate and transdifferentiate towards activated myofibroblasts.

In contrast, decreased numbers of Glut1 molecules are reported in hepatocytes subjected to chronic alcohol consumption[27] resulting in a reduced availability of glucose for glycolysis in hepatocytes. The resulting energy deficiency has been shown to impair this cell type's ability to perform critical functions and to contribute therefore to cell death and alcoholic liver disease.

In line with our results, relations between glucose metabolism and fibroproliferative processes were identified in other organs. For example in human kidney[28,29] exposure of proximal tubule cells and cortical fibroblasts to high extracellular glucose concentrations results directly in altered cell growth and collagen synthesis.

IGFBP3 and Cyp2E1, known to participate in glucose metabolism were upregulated in AdSmad7 infected HSCs, indicating that they might be under negative control through TGF-β. However, upregulation of IGFBP3 in our study could be simply due to "culture" but not "*in vivo* activation" of HSCs (for term definition compare[14]) instead of being mechanistically important. Interestingly, Smad7 even seems to enhance the upregulation of IGFBP3, which already occurs upon HSC activation. If there is any pathologic relevance of this finding, it suggests a mechanism of regulation independent of canonical TGF-β/Smad7 signaling in HSC activation.

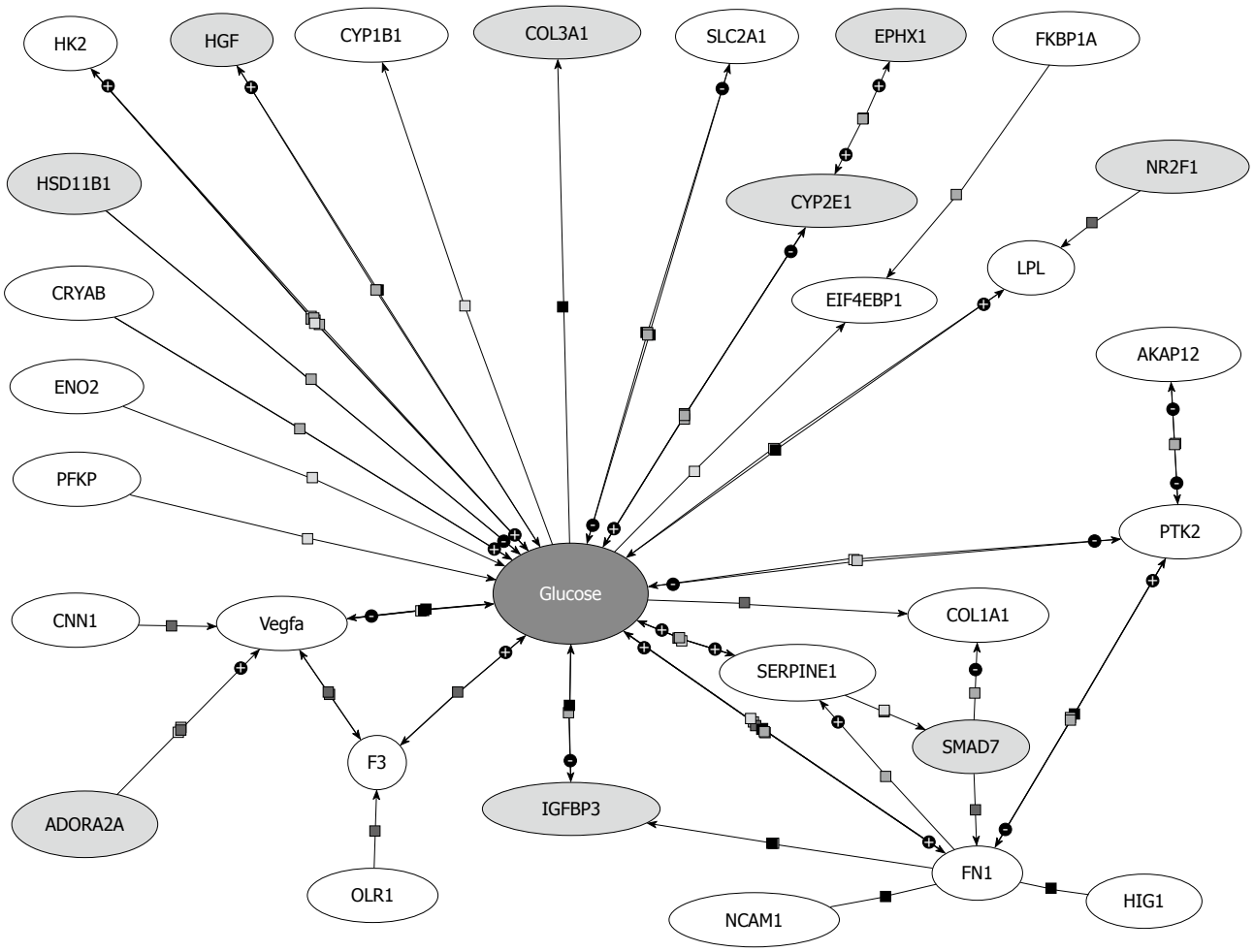


Figure 6 Biological interaction between glucose and genes up- (light grey) or down-regulated (white) in primary hepatic stellate cells after overexpression of Smad7. Genes linked to glucose by binding or regulatory interactions are depicted as interconnecting lines between glucose and the gene symbols. Pathway analysis was done with Pathway Architect software (Stratagene).

VEGFs are growth factors involved in angiogenesis, vasculogenesis and endothelial cell growth, promotion of cell migration, apoptosis inhibition and induction of blood vessel permeabilization. De Minicis *et al*[14] showed VEGFc to be upregulated[14], while in the present report VEGFa was downregulated after Smad7 overexpression in activated HSCs, indicating its induction as a response to profibrogenic TGF-β signaling. Previous investigation of hypoxia in a stellate cell line demonstrates upregulation of VEGF expression[30]. Hypoxia leads to cell dysfunction or death and occurs during liver damage and inflammation. HIF1, considered to be the major regulator of about 100 genes including VEGF and PAI-1, is also upregulated in that study. In contrast HIF1 did not display an altered expression in our study indicating that HIF1 expression in HSCs is TGF-β independent and that another TGF-β dependent route exists to induce PAI-1 (serpine1) and VEGFa expression.

Smad7 decreases cell adhesion regulators in HSCs

Expression of several proteins linked to cell adhesion, i.e. Olr1, Ncam1, Fn1 and Tnc was decreased after Smad7 overexpression in HSCs. Accordingly, Ncam1 and Fn1 were upregulated in activated HSCs[14]. Although for Olr1 no information about the regulation in activated HSCs is available so far, we assume from our results that Smad7 antagonizes cell adhesion features of activated HSCs. This suggests that profibrogenic TGF-β signaling improves cell adhesion for transdifferentiating HSCs. Nevertheless it should be noted that downregulation of Tnc in activated HSCs is supported by Smad7.

Smad7 induces BMP2 expression in HSCs

Generally, TGF-β signals *via* Smad2 and Smad3 but also induces the second canonical pathway *via* ALK1/Smad1/5/8. BMP2, another member of the TGF-β family, solely signals *via* Smads1/5/8 utilizing ALK3 and ALK6[12]. Here we show that (1) BMP2 was strongly upregulated in Smad7 expressing HSCs (Table 2, Figure 4A and B); and (2) Smad7 potently inhibited BMP2 dependent and TGF-β dependent Smad1 phosphorylation. Even in fully activated HSCs which are not responsive to TGF-β concerning Smad1 phosphorylation, BMP2 dependent Smad1 phosphorylation was abolished (Figure 5).

These results indicate a tight crosstalk between TGF-β and BMP signaling pathways in HSCs. It seems that HSCs

try to keep up a functional Smad1 signaling upon blocking TGF-β pathways with Smad7. Accordingly, BMP2 is capable of inducing Smad1 signaling in fully activated 7 d-HSCs which are no longer responsive to TGF-β stimulation. Thus BMP2 expression might be induced to overcome a lack of TGF-β/Smad1 signaling upon Smad7 expression or HSC activation using a corresponding autocrine loop. Although our *in vitro* experiments demonstrate that Smad7 is able to inhibit BMP2/Smad1 signaling effectively, Smad7 dependent induction of BMP2 expression in HSCs *in vivo* might be strong enough to sustain an active Smad1/5/8 signaling pathway. Further experiments could delineate whether BMP2 expression is directly induced by the recently described DNA binding activity of Smad7[31], if a running TGF-β signaling pathway has a negative regulatory role toward the BMP2 promoter or if other mechanisms are responsible for BMP2 induction in Smad7 overexpressing HSCs.

In summary we conclude that genes regulated contrarily during HSC activation[14] *vs* ectopic Smad7 expression (this study) most probably represent critical profibrogenic components. As Smad7 is able to blunt HSC transdifferentiation *in vitro* and *in vivo*[7] we assume glucose metabolism and the crosstalk between the TGF-β and the BMP2 pathways are critical components of HSC activation.

In general our study underlines the potential of top down systemic approaches to delineate effects of cell signaling regulation and opens the opportunity to find targets for drug development.

COMMENTS

Background

Activation of hepatic stellate cells (HSCs) as a consequence of liver damage includes proliferation and extracellular matrix (ECM) deposition and represents a major step in fibrogenesis. Transforming growth factor (TGF)-β is a master contributor and its signaling pathway is modulated during the HSC activation process, whereby its cytostatic action is lost and ECM producing effects become predominant. Smad7 is a powerful antagonist of TGF-β. Expression of Smad7 is transiently induced by the canonical TGF-β/R-Smad signaling cascade, thereby providing a negative feedback loop to regulate TGF-β signals. Smad7 is able to inhibit HSC transdifferentiation and attenuate the extent of fibrosis, suggesting Smad7 is a promising antifibrotic compound.

Research frontiers

The findings offer important new information about the process of HSC transdifferentiation and fibrogenesis as well as cell biology of signal transduction in the liver. Moreover, providing a list of genes previously not known as participants in HSC activation and fibrogenesis, members of the field may use these data as starting points to get new insight into mechanisms of HSC (patho)physiology. Thus, it will definitely be of interest to the scientific community, especially in the field of hepatology and gastroenterology.

Innovations and breakthroughs

In the present report, the authors systematically investigated transcriptional effects of Smad7 overexpression in cultured HSCs by microarray analysis. Using this powerful top down approach, the authors identified 100 target genes to be significantly regulated by Smad7 overexpression. These represent potential targets for delineating mechanisms of HSC activation and to set up therapeutic approaches. The results imply a crosstalk between TGF-β and BMP2 signaling pathways in HSCs and for the first time a significant involvement of glucose metabolism in the HSC transdifferentiation processes.

Applications

The results are of special interest for future attempts to understand the process of stellate cell activation and to set up TGF-β and/or Smad7 directed treatment approaches in chronic liver diseases, especially as they reflect the most powerful negative regulatory process of TGF-β signaling.

Terminology

Bone morphogenetic protein (BMP): BMPs are multi-functional growth factors belonging to the TGF-β superfamily. BMPs were originally discovered by their ability to induce the formation of bone and cartilage, but are now considered to constitute a group of pivotal morphogenetic signals, orchestrating tissue architecture throughout the body. BMP signals are mediated by type Ⅰ and Ⅱ BMP receptors and their downstream molecules Smad1, 5 and 8. Microarray: A method for profiling gene and protein expression in cells and tissues. A microarray consists of different nucleic acid/protein probes that are chemically attached to a substrate, which can be a microchip, a glass slide or a microsphere-sized bead. Hybridization of test samples to these probes can be measured by different means.

Peer review

This is a potentially interesting study aimed at clarifying Smad7-regulated gene expression during the transdifferentiation of hepatic stellate cells, a major profibrogenic cell type in the liver. Overall, the study is well-performed and the manuscript is well-written.

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BRIEF ARTICLE

Transient elastography: A non-invasive tool for assessing liver fibrosis in HIV/HCV patients

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Abstract

AIM: To assess the prevalence of advanced liver fibrosis (ALF) in human immunodeficiency virus (HIV), hepatitis C virus (HCV) and HIV/HCV patients using transient elastography, and to identify factors associated with ALF.

METHODS: Between September 2008 and October 2009, 71 HIV mono-infected, 57 HIV/HCV co-infected and 53 HCV mono-infected patients on regular follow-up at our Center were enrolled in this study. Alcohol intake, the main parameters of liver function, presence of HCV-RNA, HIV-RNA, duration of highly active anti-retroviral therapy (HAART) and CD4 cell count were recorded. ALF was defined as liver stiffness (LS) $\geqslant$ 9.5 kPa. To estimate liver fibrosis (LF) a further 2 reliable biochemical scores, aspartate aminotransferase platelet ratio index (APRI) and FIB-4, were also used.

RESULTS: LS values of co-infected patients were higher than in either HIV or HCV mono-infected patients (χ^2_{MH} = 4, P < 0.04). In fact, LS $\geqslant$ 9.5 was significantly higher in co-infected than in HIV and HCV mono-infected patients (χ^2 = 5, P < 0.03). Also APRI and the FIB-4 index showed more LF in co-infected than in HIV mono-infected patients (P < 0.0001), but not in HCV mono-infected patients. In HIV/HCV co-infected patients, the extent of LS was significantly associated with alcohol intake (P < 0.04) and lower CD4+ cell count (P < 0.02). In HCV patients, LS was correlated with alcohol intake (P < 0.001) and cholesterol levels (P < 0.03). Body mass index, diabetes, HCV- and HIV-viremia were not significantly correlated with LS. In addition, 20% of co-infected patients had virologically unsuccessful HAART; in 50% compliance was low, CD4+ levels were < 400 cells/mm^3 and LS was > 9.5 kPa. There was no significant correlation between extent of LF and HAART exposure or duration of HAART exposure, in particular with specific dideoxynucleoside analogues.

CONCLUSION: ALF was more frequent in co-infected than mono-infected patients. This result correlated with lower CD4 levels. Protective immunological effects of HAART on LF progression outweigh its hepatotoxic effects.

Key words: Liver fibrosis; Transient elastography; Aspartate aminotransferase platelet ratio index; FIB-4 test; Fibrosis evaluation; Human immunodeficiency virus infection; Hepatitis C virus infection

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INTRODUCTION

In the last few years, liver disease associated with hepatitis C virus (HCV) has emerged as a significant problem in human immunodeficiency virus (HIV)-infected patients, thanks to improved survival in the highly active antiretroviral therapy (HAART) era[1]. It has been reported that HIV and HCV co-infection leads to a more rapid progression of liver disease to cirrhosis[2,3]. Other factors such as severe immune suppression and alcohol consumption accelerate the progression of HCV-related fibrosis[4,5]. Virologically successful HAART slows the progression of liver fibrosis (LF) and reduces hepatic necroinflammatory activity in HIV/HCV co-infected patients[2,6]. In contrast, antiretroviral-related liver toxicity could contribute to liver damage in HIV- and HIV/HCV-infected patients[7]. Mitochondrial toxicity of nucleoside analogues[8], and glucose or lipid abnormalities, such as hyperglycemia and lipodystrophy, which are particularly common when using some protease inhibitors[9], may produce or enhance LF progression in HIV mono- and HIV/HCV co-infected patients. Currently, in this respect, a growing number of cases of cryptogenetic liver disease in symptomatic and asymptomatic HIV-infected patients has been reported[10,11].

Percutaneous liver biopsy is the gold standard for assessing LF. However, it may be associated with sampling variability[12], is an invasive technique with rates of morbidity of 3% and mortality of 0.03%[13,14], and as a consequence, is not suitable for repeated assessment, which is required when monitoring LF.

For these reasons, new non-invasive methods for the assessment of LF have been developed. Transient elastography (TE) (Fibro-Scan®; EchoSens, Paris, France) is a rapid, reliable and tolerable imaging technique for the assessment of LF by measuring liver stiffness (LS)[15,16].

On the other hand, many biochemical markers have been implemented to estimate LF, with the aim of reducing the number of liver biopsies[14].

The advent of TE and biochemical markers has been demonstrated to be very helpful in the non-invasive measurement of LF, particularly in asymptomatic HIV-infected patients in whom liver biopsy is not recommended[11]. TE has already been validated for the measurement of LF in HIV and HCV seropositive patients[17,18].

The aim of this study was to assess the prevalence of LF and cirrhosis in a group of HIV mono-infected, HCV mono-infected and HIV/HCV co-infected patients using TE and biochemical markers. In addition, we evaluated which of the factors studied correlated with advanced LF (ALF) and cirrhosis.

MATERIALS AND METHODS

Study population

Between September 2008 and October 2009 all consecutive HIV mono-infected and HIV/HCV co-infected patients on regular follow-up at the AIDS Center of the University of Palermo, as well as HCV mono-infected patients seen consecutively at the Outpatient Clinic of the Department of Clinical Medicine and Emerging Pathologies of the University of Palermo were enrolled in this study.

Information on age, gender, risk factors for HCV and HIV infections, cumulative exposure to non-nucleoside and nucleoside reverse-transcriptase inhibitors, protease inhibitors and specific antiretroviral drugs within each class were all recorded in a database designed for this study.

For all HIV-infected patients the absolute number of CD4+ T-cells and plasma HIV-RNA levels was assessed. In HCV-infected patients, HCV-genotype and plasma HCV-RNA levels were also recorded. In addition, at baseline, complete blood cell counts, alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transferase (γGT), total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides and glycemia were measured.

Alcohol intake > 20 g/d either at the time of the study or in the past was recorded through patient interviews. Diabetes or impaired fasting glucose (IFG) were defined according to the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus criteria[19].

Patients with acute liver decompensation, hepatocellular carcinoma or chronic hepatitis B were excluded.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Patients were enrolled after written informed consent was obtained.

Assessment of LF

LF was assessed by a single certified operator (trained by the manufacturer) using TE (FibroScan®; EchoSens, Paris, France). TE provides an assessment of LS expressed in kPa units. In brief, an ultrasound transducer probe is mounted on the axis of a vibrator. Vibrations of mild amplitude and low frequency are transmitted by the transducer, inducing an elastic shear wave that propagates through the underlying tissues. The speed of propagation of this vibration across the liver is directly related to tissue stiffness.

The tip of the probe transducer was placed in the intercostal spaces at the right lobe of the liver. Only patients with 10 valid elastometric measures, interquartile ranges > 30% and ⩾ 60% success rate (the number of validated

measurements divided by the total number of measurements) were considered to be reliable. ALF (severe fibrosis and cirrhosis) was defined as a median LS of 9.5 kPa. As previously published, this cut-off value is strongly correlated with a Metavir score of F3, both in HCV mono-infected and HCV/HIV co-infected patients[17,18].

LF was also assessed biologically using 2 different well-validated indices, the aspartate aminotransferase platelet ratio index (APRI) index and the FIB-4 index. The APRI was calculated as follows: AST/upper limit of normal × 100/platelet count (10^9/L)[20,21]. The FIB-4 index was calculated as follows: age × AST (IU/L)/{[platelet count (10^9/L)] × [ALT (IU/L)]$^{1/2}$}[22]. The prevalence of ALF was estimated using as a reference a FIB-4 index > 3.25 and an APRI index > 1.5[20,22].

Statistical analysis

When data distribution was Gaussian, values were expressed as mean ± SD and their differences were calculated using the Student *t*-test; otherwise, data were expressed as the median and range and analyzed using the Mann-Whitney *U* test. Fisher's exact and χ^2 tests, the χ^2 test of Mantel Haenszel, Spearman's rank correlations (ρ) and Pearson's correlation (*r*) were used where appropriate. Multiple linear regression analysis was used to study the association between increased values of LS and variables statistically significant at univariate analysis. All analyses were performed using the SPSS software package (version 13.0; Chicago, IL, USA).

RESULTS

Study population

A total of 201 patients on regular follow-up at both our Centers were enrolled in the study.

In 11 patients (4 HIV mono-infected, 6 HCV mono-infected and one co-infected) a valid elastometric assessment could not be obtained because of truncular obesity, therefore 190 patients were finally included in this study. There were 137 HIV-infected patients, including 71 HIV mono-infected and 66 HIV/HCV co-infected, and 53 HCV mono-infected patients. Patient characteristics at the time of LS measurement are summarized in Table 1.

HIV patients were significantly younger than HCV mono-infected individuals ($P < 0.002$). Body mass index (BMI) was significantly higher in HCV mono-infected patients than in HIV and HIV/HCV co-infected patients ($P < 0.002$). The most frequent risk factor of HCV contamination was intravenous drug use in co-infected *vs* mono-infected HCV patients ($P < 0.0001$), while it was transfusion of blood products in HCV mono-infected *vs* co-infected patients ($P < 0.0001$). Most HIV mono-infected (80.2%) and HIV/HCV co-infected patients (90.9%) were under HAART. However, only 62% of HIV mono-infected and 68% of HIV/HCV co-infected patients had an HIV-RNA load of < 47 copies/mL. In addition, 20% of co-infected patients had virologically unsuccessful HAART; in 50% compliance was low, and CD4+ levels were < 400 cells/mm^3. HIV/HCV co-infected patients were more often infected by HCV genotype 3 compared with HCV mono-infected patients ($P < 0.03$).

Table 1 Demographic characteristics of the study populations (mean ± SD) *n* (%)

	HIV (*n* = 71)	HCV (*n* = 53)	HIV/HCV (*n* = 66)
Age (yr)	43.3 ± 10.3[a]	49.7 ± 12.5	45.6 ± 12.5
Male gender	50 (70)	36 (67.9)	49 (74.2)
BMI (kg/m^2)	23.6 ± 3.4[a]	26.0 ± 3.6	22.8 ± 3.0[b]
Risk group			
Transfusion	-	17 (32.1)	4 (6.1)[b]
Homosexual men	21 (29.5)	-	1 (1.5)
Intravenous drug use	4 (5.6)	7 (13.2)[c]	41 (62.1)
Others	-	12 (22.6)	-
Unknown	9 (12.6)	16 (30.2)	8 (12.1)
HCV genotype 3:non3	-	7:46[d]	20:46
HAART	57 (80.2)	-	60 (90.9)
Alcohol	6 (8.4)	4 (7.5)	5 (7.5)
Diabetes + IFG	2 (2.8)	4 (7.5)[e]	11 (16.6)

[a]$P < 0.002$, [b]$P < 0.0001$ *vs* hepatitis C virus (HCV); [c]$P < 0.0001$, [d]$P < 0.03$, [e]$P < 0.001$ *vs* human immunodeficiency virus (HIV)/HCV. IFG: Impaired fasting glucose; BMI: Body mass index; HAART: Highly active anti-retroviral therapy.

Table 2 shows the main hematological and virological parameters in the 3 study groups. Serum ALT levels were significantly higher in HCV mono-infected patients than in HIV/HCV co-infected and HIV mono-infected patients ($P < 0.02$). Serum AST, ALT and γGT levels were significantly higher in HIV/HCV co-infected than in HIV mono-infected patients ($P < 0.0001$). Only γGT levels were more elevated in HIV/HCV co-infected patients than in HCV mono-infected patients ($P < 0.01$). TG levels were significantly higher in HIV mono-infected and HIV/HCV co-infected patients than in HCV mono-infected patients ($P < 0.004$).

Extent of LF

In the overall population LS measured by TE ranged from 3.2 to 48.8 kPa. In 9 HIV/HCV co-infected patients, HCV-RNA was undetectable and for this reason these patients were excluded from the analysis, which was thus carried out only in the remaining 57 co-infected patients. However, their LS was lower than in the remaining co-infected group (data not shown).

Table 3 shows the distribution of LS values measured in all 3 study groups. Co-infected patients had higher LS values than mono-infected patients ($\chi^2_{MH} = 4$, $P < 0.04$). The HIV/HCV-co-infected population had LS ≥ 9.5 kPa (50.9%) more often than HCV and HIV mono-infected patients (28.3%) ($\chi^2 = 5$, $P < 0.03$). In this respect 60% of co-infected patients under virologically unsuccessful HAART showed LS ≥ F3. The individual values of LS increased from HIV to HCV and to HIV/HCV infected patients (ρ = 0.5, $P < 0.0001$) (Figure 1).

Overall, by multiple linear regression analysis, the variables significantly associated with ALF were AST values (β = 0.47, $P < 0.0001$) and HIV/HCV co-infection (β = 0.25, $P < 0.002$). To better understand which variables

Table 2 Main hematological and virological parameters in the three study groups (mean ± SD)

	HIV (*n* = 71)	HCV (*n* = 53)	HIV/HCV (*n* = 66)
AST/ALT	1 ± 0.4	0.72 ± 0.28[a,j]	0.9 ± 0.4
ALT (U/L), mean (range)	22 (7-113)[f,h]	75 (16-551)	56.5 (9-280)[g]
AST (U/L), mean (range)	21 (11-56)[f,h]	52.5 (18-209)	42.5 (14-281)
γGT (IU/L), mean (range)	33 (9-461)[h]	40.5 (10-479)[i]	76.5 (11-479)
Mean platelet count (10^9/L)	208 ± 59	184 ± 60	173 ± 61[b]
T-Chol (mg/dL)	201 ± 46	175 ± 28[d]	171 ± 48[c]
HDL-Chol (mg/dL)	44 ± 16	47 ± 12	44 ± 15
LDL Chol (mg/dL)	123 ± 32	108 ± 37.5	97 ± 40[a]
TG (mg/dL), mean (range)	137 (36-615)	103 (32-354)[e,j]	118 (49-614)
HCV-RNA (IU/mL) > 700.000	-	29 (54.7)	44 (66.6)
HIV-RNA (copies/mL), mean (range)	6050 (50-700 000)	-	3300 (60-1 100 000)
HIV-RNA < 47 copies/mL (%)	44 (62)		45 (68.1)
CD4+ count (cells/μL), mean (range)	466.5 (17-1282)	-	446 (35-1445)
CD4+ < 200 cells/μL	38[1]	-	38[2]

[a]$P < 0.0001$, [b]$P < 0.002$, [c]$P < 0.001$, [d]$P < 0.004$, [e]$P < 0.02$ *vs* human immunodeficiency virus (HIV); [f]$P < 0.0001$, [g]$P < 0.02$ *vs* hepatitis C virus (HCV); [h]$P < 0.0001$, [i]$P < 0.01$, [j]$P < 0.05$ *vs* HIV/HCV. [1]Data available in 66 patients; [2]Data available in 56 patients. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGT: γ-glutamyl transferase; T-Chol: Total cholesterol; HDL: High density lipoprotein; LDL: Low density lipoprotein; TG: Triglyceride.

Table 3 Estimates of liver fibrosis using transient elastography in the study population *n* (%)

LS	Metavir	HIV (*n* = 71)	HCV (*n* = 53)	HIV/HCV (*n* = 57)	
< 7.1	F0-F1	56 (78.8)	23 (43.4)	19 (33.3)	
7.1-9.4	F2	11 (15.4)	15 (28.3)	9 (15.8)	
9.5-12.4	F3	1 (1.4)	4 (7.5)	9 (15.8)	
≥ 12.5	F4	3 (4.2)	11 (20.7)	20 (35.1)	$\chi^2_{MH} = 4$, $P < 0.04$
≥ F3		4 (5.6)	15 (28.3)	29 (50.9)	$\chi^2 = 5$, $P < 0.03$

HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; LS: Liver stiffness.

Table 4 Estimates of liver fibrosis using biochemical markers and transient elastography, mean (range)

	HIV (*n* = 71)	HCV (*n* = 53)	HIV/HCV (*n* = 57)
APRI	0.31 (0.1-1.6)[a,b]	0.93 (0.28-6.07)	0.92 (0.17-19.7)
FIB-4	0.93 (0.39-4.28)[a]	1.62 (0.47-12.6)	1.63 (0.63-23.8)
LS	5.4 (3.2-26.6)[a,b]	7.3 (3.4-40.9)[c]	9.8 (4.3-48.8)

[a]$P < 0.0001$ *vs* hepatitis C virus (HCV); [b]$P < 0.0001$, [c]$P < 0.05$ *vs* human immunodeficiency virus (HIV)/HCV. LS: Liver stiffness; APRI: Aspartate aminotransferase platelet ratio index.

were associated with LS in patients with HIV mono- and co-infection, we also performed multiple linear regression analysis of these 2 groups and found that ALF correlated positively with AST serum levels (β = 0.34, $P < 0.0001$) and presence of HIV/HCV co-infection (β = 0.4, $P < 0.0001$) and negatively with lower CD4+ cell counts (β = -0.21, $P < 0.003$).

Median values of APRI and FIB-4 were significantly higher in HCV mono- and co-infected patients than in HIV mono-infected patients. There were no significant differences in APRI and FIB-4 medians in HCV mono- and HIV/HCV co-infected patients (Table 4). Median values of LS were significantly lower in HIV mono-infected than in HCV mono- and HIV/HCV co-infected patients ($P < 0.0001$). Median values of HIV/HCV co-infected patients were also significantly higher than in HCV mono-infected patients ($P < 0.05$).

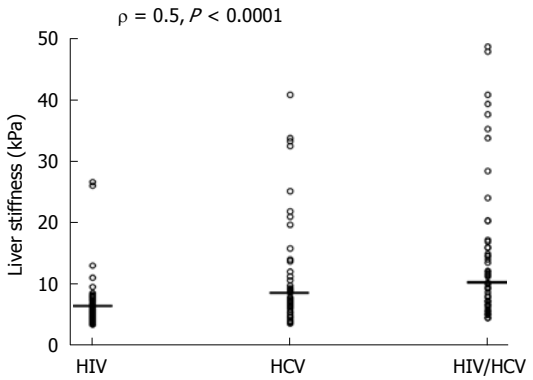

Figure 1 Median and distribution of liver stiffness in human immunodeficiency virus, hepatitis C virus, human immunodeficiency virus/hepatitis C virus patients. HIV: Human immunodeficiency virus; HCV: Hepatitis C virus.

Overall, correlations between LS and APRI values and LS and FIB-4 values were statistically significant ($r = 0.60$, $P < 0.0001$ and $r = 0.64$, $P < 0.0001$, respectively). However, when correlations were made according to the 3 cut-off values of LS, we found a significant correlation only for values of LS ≥ 9.5 ($r = 0.50$, $P < 0.0001$ *vs* APRI and $r = 0.53$, $P < 0.0001$ *vs* FIB-4) (Table 5).

Correlation between LS and risk factors studied

Table 6 shows the factors associated with LS in the 3 groups. No correlation between the studied parameters and LS was found in the HIV mono-infected group. In HCV mono-infected patients, LS positively correlated with alcohol intake > 20 g/d ($P < 0.001$) and AST serum level ($P < 0.0001$), while it negatively correlated with the

Table 5 Correlation between liver stiffness and aminotransferase platelet ratio index/FIB-4 values according to the liver stiffness cut-offs

	LS < 7.1 kPa (*n* = 98)		LS (7.1-9.4 kPa) (*n* = 35)		LS ≥ 9.5 kPa (*n* = 48)	
	r	*P*	*r*	*P*	*r*	*P*
APRI	0.01	NS	0.23	NS	0.50	< 0.0001
FIB-4	0.01	NS	0.25	NS	0.53	< 0.0001

APRI: Aminotransferase platelet ratio index; LS: Liver stiffness; NS: Not significant.

Table 6 Correlation (*r* or ρ) between liver stiffness and studied parameters

	LS		
	HIV	HCV	HIV/HCV
BMI	0.11	-0.1	0.02
HCV genotype 3/non 3	-	-0.1	-0.26
HCV-RNA	-	-	-0.26
HIV-RNA	0.21	-	0.18
Time HAART	-0.047	-	0.09
CD4 cells count	0.074	-	-0.32[d]
Alcohol	-0.096	0.70[a]	0.30[e]
T-Chol	-0.09	-0.32[b]	-0.18
TG	0.06	0.18	0.1
HDL-Chol	-0.1	0.05	-0.16
Diabetes + IFG	-0.01	0.19	0.14
Time HCV	-	0.24	-0.02
ALT	0.03	0.1	0.23
AST	0.18	0.56[c]	0.40[a]
Platelets	-0.02	-0.56[c]	-0.61[c]
APRI score	0.04	0.70[c]	0.50[c]
FIB-4 score	0.06	0.75[c]	0.60[c]

LS: Liver stiffness; HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; BMI: Body mass index; HAART: Highly active anti-retroviral therapy; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; T-Chol: Total cholesterol; HDL: High density lipoprotein; TG: Triglyceride; IFG: Impaired fasting glucose; APRI: Aminotransferase platelet ratio index. [a]$P < 0.001$; [b]$P < 0.03$; [c]$P < 0.0001$; [d]$P < 0.02$; [e]$P < 0.04$.

number of platelets ($P < 0.0001$) and serum cholesterol levels ($P < 0.03$). APRI and FIB-4 values were significantly associated with LS both in HCV and HIV/HCV co-infected patients ($P < 0.0001$).

In HIV/HCV-co-infected patients the extent of LS was significantly correlated with alcohol intake ($P < 0.04$) AST level ($P < 0.0001$) and lower CD4+ cells count ($P < 0.02$) and was negatively correlated with platelets ($P < 0.0001$).

BMI, presence of diabetes or IFG, HCV- and HIV-viremia were not significantly correlated with LS in any of the 3 study groups. In addition, there was no significant correlation between the extent of LF under HAART exposure and duration of HAART exposure. There was no significant correlation either between extent of LF and cumulative exposure to each class of antiretroviral drugs (non-nucleoside/nucleoside reverse-transcriptase inhibitors, protease inhibitors) and of specific dideoxynucleoside analogues (didanosine, stavudine, zidovudine).

DISCUSSION

Our results, in agreement with other studies, confirmed that LF is more severe in HIV/HCV co-infected patients than in HCV or HIV mono-infected patients[5,23,24]. In addition, ALF was significantly associated with a lower CD4+cell count in co-infected patients. There is convincing evidence that co-infection with HIV worsens the prognosis of HCV-related liver disease. It has been reported that in patients co-infected with HIV and HCV the risk of progressing to cirrhosis and liver failure is higher than in those infected with only HCV[25,26], especially in individuals with CD4 < 200 cells/μL and alcohol consumption[2].

In our study, 20% of HIV/HCV co-infected patients were under virologically unsuccessful HAART and in 50% CD4+ levels were below 400 cells/mm^3 suggesting, in agreement with former findings, that the less successful the response to HAART, the less marked is its clinical benefit. In fact, immune recovery under HAART has been associated with longer overall survival, slower progression of HCV-related liver damage in HIV co-infected patients and with lengthier survival times before death attributable to liver disease[27]. In the same way, Pineda *et al*[27] demonstrated that liver decompensation emerged earlier in patients who maintained an undetectable HIV viral load for a shorter period during follow-up. Nevertheless, the association between ALF and lower CD4 cell count suggests that the response to HAART, measured using CD4 cell gain and HIV viral load decline, determines the evolution of liver disease and that virologically and immunologically successful HAART may slow progression of LF in HIV/HCV co-infected patients.

On the other hand, antiretroviral-related liver toxicity could have further contributed to liver damage in our HIV population[7]. Mitochondrial toxicity of nucleoside analogues and glucose or lipid abnormalities, particularly common when using some protease inhibitors, may produce or enhance LF progression in HIV-seropositive patients. The correlation between use of antiretroviral drugs and LF in patients with HIV/HCV co-infection has been evaluated in different studies but with contradictory results[21,28-30].

Macías *et al*[21] reported that HAART regimens, including nevirapine, were associated with an increased degree of LF, while the use of protease inhibitor-based HAART was associated with less severe fibrosis and a slower progression of fibrosis in HIV/HCV co-infected patients. In contrast, Berenguer *et al*[28] found that exposure to NNRTI was associated with a reduction in LF progression. In addition, Halfon *et al*[29] showed that exposure to NNRTI was an independent factor in LF while Blanco *et al*[30] highlighted that exposure to dideoxynucleosides was an independent factor associated with ALF.

In our study, no correlation was found between HAART exposure, duration of HAART exposure or cumulative exposure to any class of antiretroviral drugs and LF. In addition, we analyzed the correlation between duration of

exposure to dideoxynucleosides (in particular didanosine, stavudine, zidovudine) and LF but also in this case no correlation was observed, suggesting that these drugs could not play any role in the progression of LF.

ALF was significantly higher in HIV/HCV co-infected patients than in HCV mono-infected patients when the subset of co-infected patients with undetectable HCV-RNA were excluded from the analysis. Overall, HIV/HCV co-infected patients with undetectable HCV-RNA had either no or only mild fibrosis (F0-F1) compared to the remaining co-infected patients, suggesting that the presence of HCV is important in conditioning the progression of LF and that anti-HCV therapy is mandatory in HIV/HCV co-infected patients in order to eradicate the virus. In fact, other authors have reported that achieving HCV clearance may reduce liver-related complications and mortality[31,32] and probably permits at least a partial regression of LF[33]. However, in HIV-positive patients liver cirrhosis may also occur without chronic viral hepatitis, and possible causes of hepatic steatosis in patients with HIV may be due to HIV itself, pathological alcohol use, diabetes mellitus, obesity or antiretroviral medications[34].

Evaluation of LF using the 2 biochemical scores (APRI and FIB-4) was not in full agreement with LS measurement. In fact, these 2 biochemical tests were in agreement with TE values only for high-grade LF, but not in low and moderate LF, suggesting that at least in these cases liver biopsy could be necessary to assess the precise degree of LF. In this respect, we are aware that the lack of liver biopsy, as a reference tool of LF, is a limitation of our study.

More consideration, perhaps, should be given to transaminase levels. In fact, the HCV mono-infected group had the highest levels of transaminases, which may have influenced LS values by increasing them. This result could further support our observation that co-infected patients are at highest risk of LF because of their high AST levels and the immune suppression associated with a low CD4 cell count.

Finally, also in our area, HCV genotype 3 was confirmed to be more associated with HIV-positive patients, because of their habits as drug abusers[35].

In conclusion, in our population, HIV/HCV co-infected patients had more ALF than HCV and HIV mono-infected patients. This result was not correlated with long-term exposure to HAART but with a lower CD4 cell count, suggesting that immunologically successful HAART may protect from progression of liver damage in HIV/HCV co-infected patients. In addition, the detection of unsuspected ALF in HIV mono-infected patients confirms that FibroScan® is very useful in this population. HCV infection, with its different pattern of cytolysis, may condition LS values, but viral eradication is mandatory to reduce fibrosis progression. Finally, the use of these non-invasive parameters of LF should be considered with caution. In fact, from our data it emerges that both TE and the biochemical scores may be suitable only for high grades of LF. In contrast, for mild/moderate degrees of fibrosis, they could not replace liver biopsy in the correct evaluation of LF.

COMMENTS

Background

Liver disease associated with hepatitis C virus (HCV) has emerged as a significant problem in human immunodeficiency virus (HIV) patients, thanks to improved survival in the highly active anti-retroviral therapy (HAART) era. Co-infection with HIV is known to lead to a more rapid progression of HCV liver disease to cirrhosis. Other factors such as severe immune suppression and alcohol consumption accelerate the progression of HCV-related fibrosis. In addition, successful HAART slows the progression of liver fibrosis (LF), but antiretroviral-liver toxicity could contributes to hepatic damage in co-infected patients. The advent of transient elastography (TE) has demonstrated to be very helpful for the non-invasive measurement of LF.

Research frontiers

Percutaneous liver biopsy is the gold standard for assessing LF but it is an invasive technique with risk of morbidity and mortality. For these reasons new non-invasive methods for the assessment of LF have recently been developed. TE (FibroScan®) and biochemical markers have demonstrated to be very helpful in the non-invasive measurement of LF. In this study, using these non-invasive tools, i.e. TE plus 2 biochemical tests, aminotransferase platelet ratio index (APRI) and FIB-4, we showed that advanced LF was significantly higher in HIV/HCV co-infected patients than in mono-infected patients and that it was significantly associated with lower CD4+ cells count. The APRI and FIB-4 tests correlated only with the highest values of TE, i.e. $\geqslant$ 9.5, suggesting that they are useful tools in diagnosing high grade liver disease, but in the case of a low or moderate degrees of LF liver biopsy remains the best means for correctly diagnosing the degree of fibrosis. Furthermore, the results showed that, overall, a greater number of HIV/HCV co-infected patients with undetectable HCV-RNA had either no or mild fibrosis (F0-F1) compared to the remaining co-infected patients with detectable HCV-RNA.

Innovations and breakthroughs

Several studies have been carried out to identify factors related to an accelerated progression of LF in HIV/HCV co-infected patients. Conflicting results have been reported in the literature about the role of antiretroviral therapy on the progression of LF. In the study, information on alcohol intake, duration of HCV infection and cumulative exposure to non-nucleoside and nucleoside reverse-transcriptase inhibitors, protease inhibitors and specific dideoxynucleoside analogues (didanosine, stavudine, zidovudine) was evaluated. The results showed that on univariate analysis liver stiffness (LS) was significantly associated with alcohol intake > 20 g/d in both HCV mono-infected and co-infected patients, but we did not find any correlations between LF and duration of HCV infection, HAART exposure, duration of HAART exposure or cumulative exposure to any class of antiretroviral drugs.

Applications

A good adherence to antiretroviral therapy, when it is indicated, is important to reduce the risk of progression of LF in co-infected patients. In addition, HCV mono- and co-infected patients should modify negative habits and lifestyles, such as alcohol consumption, which could accelerate the progression of LF. Important fields for further study could include the use and evaluation of the applicability of FibroScan® for repeated assessment in the monitoring of LF.

Terminology

Transient elastometry (Fibro-Scan®; EchoSens, Paris, France) is a rapid, reliable and well-tolerated imaging technique for the assessment of LF by measuring LS.

Peer review

The authors aimed to assess the prevalence of advanced LF (ALF) in HIV, HCV and HIV/HCV patients using TE and to identify factors associated with ALF. They concluded that HIV/HCV co-infected patients had ALF more frequently at TE than HCV and HIV mono-infected patients. The title reflects accurately the contents of the article, and the abstract delineates concisely the research.

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BRIEF ARTICLE

NKX2-3 and IRGM variants are associated with disease susceptibility to IBD in Eastern European patients

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Abstract

AIM: To investigate variants of immunity-related GTPase family M (IRGM) and NKX2-3 genes and genotype-phenotype in Eastern European patients with inflammatory bowel disease (IBD).

METHODS: We analyzed 1707 Hungarian and Czech subjects with Crohn's disease (CD) (n = 810, age: 37.1 ± 12.6 years, duration: 10.7 ± 8.4 years) and ulcerative colitis (UC) (n = 428, age: 43.7 ± 15.0 years, duration: 12.6 ± 9.9 years), as well as 469 healthy controls. IRGM rs13361189, NKX2-3 rs10883365 and ECM1 rs13294 polymorphisms were tested by LightCycler allele discrimination. Detailed clinical phenotypes were determined by reviewing the medical charts.

RESULTS: NKX2-3 rs10883365 variant allele was associated with increased risk for CD (P = 0.009, OR = 1.24, 95% CI = 1.06-1.48) and UC (P = 0.001, OR = 1.36, 95% CI = 1.13-1.63), whereas variant IRGM allele increased risk for CD (P = 0.029, OR = 1.36, 95% CI = 1.03-1.79). In contrast, ECM1 rs13294 was not associated with either CD or UC. In CD, the variant IRGM allele was associated with a colon-only location (P = 0.02, OR = 1.62, 95% CI = 1.07-2.44), whereas in UC, the ECM1 variant was associated with cutaneous manifestations (P = 0.002, OR = 3.36, 95% CI = 1.48-7.63). Variant alleles did not predict resistance to steroids or azathioprine, efficacy of infliximab, or need for surgery.

CONCLUSION: NKX2-3 and IRGM are susceptibility loci

for IBD in Eastern European patients. Further studies are needed to confirm the reported phenotype-genotype associations.

Key words: Crohn's disease; Ulcerative colitis; NKX2-3; Immunity-related GTPase family M; ECM1; Genotype; Phenotype; Pharmacogenetics

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INTRODUCTION

Inflammatory bowel diseases (IBDs) are multifactorial with both environmental and genetic components; the latter displaying heterogeneity in terms of disease presentation as well as response to treatment[1]. Crohn's disease (CD) has a strong genetic component, and to date, at least nine susceptibility loci have been identified[2]. The first, and most consistently replicated critical mutations have been found in the NOD2/CARD15 gene on chromosome 16 (IBD1). Other loci encode genes that are involved in a number of homeostatic mechanisms: innate pattern recognition receptors (including NOD2/CARD15, TLR4 and CARD9), differentiation of Th17-lymphocytes (IL-23R, JAK2, STAT3, CCR6 and ICOSLG), autophagy [ATG16L1, immunity-related GTPase family M (IRGM) and LRRK2], maintenance of epithelial barrier integrity (IBD5, DLG5, PTGER4, ITLN1, DMBT1 and XBP1), and orchestration of the secondary immune response (HLA-region, TNFSF15/TL1A, IRF5, PTPN2, PTPN22, NKX2-3, IL-12B, IL-18RAP and MST1)[3-6]. As the incidence of IBD is rapidly increasing in some parts of Eastern Europe[1], it is of great importance to study social and environmental, as well as host-genetic factors that might underlie this trend.

The IRGM gene is located on chromosome 5q33.1, and encodes an autophagy-inducing protein and belongs to the immunity-related guanosine triphosphatases (IRGs), also known as p47 guanosine triphosphatases. IRGs play an important role in host defense against intracellular pathogens[7]. Genetic association of the autophagy genes, autophagy-related 16-like 1 (ATG16L1) and IRGM has been suggested in adult-onset CD by genome-wide association scan studies (GWASs), but not in UC[5,6,8-10]. Although a meta-analysis of the three index GWASs has implicated a single nucleotide polymorphism (SNP; rs11747270) based on imputed data with an OR of 1.33[10], other studies including a new meta-analysis[11] have confirmed the IRGM signal on rs13361189 and rs4958847 immediately flanking IRGM.

Recently, the rs13361189 variant, upstream of IRGM, has been shown to be in perfect linkage disequilibrium with a 20-kb deletion polymorphism that affects the expression of IRGM (and cellular autophagy) in a tissue-specific manner[9]. These results suggest that the CD association at IRGM arises from an alteration in IRGM regulation that affects the efficacy of autophagy, and identifies rs13361189 and its strongly correlated neighbors at the 5' end of IRGM, including the 20-kb deletion polymorphism as a likely causal variant.

Association between the rs10883365 variant of NK2 transcription factor related, locus 3 (NKX2-3) gene on chromosome 10q24.2 and susceptibility of CD has been reported in Western Europe, including The Wellcome Trust Case Control Consortium (WTCCC) GWA and a replication study from The Netherlands, and in Japan, with an OR of 1.2-1.6[12-14]. In addition, a modest association ($P = 3.3 \times 10^{-4}$ in the ulcerative colitis panel and $P = 2.4 \times 10^{-6}$ using the expanded WTCCC control panel) has also been reported between rs10883365 and UC in a non-synonymous SNP scan by Fisher *et al*[15]. *NKX2-3*-deficient mice show severe defects in gut development; primarily in the epithelium of the small intestine[16]. In addition, the lymphoid organs of these mice, including the spleen and Peyer's patches, have abnormal tissue architecture and abnormalities in the migration and segregation of B and T cells[17], however, the exact mechanism is unknown.

ECM1, on chromosome 1q21.2, is also a plausible candidate gene for UC; it encodes extracellular matrix protein 1, a glycoprotein expressed in small and large intestine, and it interacts with the basement membrane and inhibits matrix metalloproteinase[18]. Notably, ECM1 strongly activates nuclear factor-κB signaling, a key immune regulator. Expression is upregulated in colorectal cancer and metastases, which implicates ECM1 in epithelial-stromal interaction[18]. Of note, the WTCCC observed modest association between these ECM1 SNPs and ankylosing spondylitis, a related inflammatory disorder ($P = 0.0041$ and 0.0044), respectively[13]. More recently, an association between the rs3737240 and rs13294 variants of ECM1 and UC susceptibility was reported in a GWAS by Fisher *et al*[15], with an OR of 1.3-1.4 for the homozygous carriage of the variant allele. rs3737240 and rs13294 encode substitutions T130M and G290S: Thr130, residing within a collagen IV binding domain, is conserved in primates, whereas Gly290 is not.

Finally, data about the pharmacogenetics of IBD are still limited. Resistance to steroids is associated with high expression of β-glucocorticoid receptors (hGRβ) and overexpression of MDR1 has been found in patients who fail steroid therapy and require surgery[19]. In contrast, according to our group's earlier results, the presence of the DLG5 Arg30Gln variant allele, but not variants in ABCG2 or MDR1 genes, predicted resistance to steroids[20,21]. Vermeire *et al*[22] have reported a lack of association between the

presence of NOD2 mutations and response to infliximab therapy, and later, the same group reported an association between the presence of Fas ligand -843C/T variant and response to infliximab[23].

In light of the lack of data in Eastern European countries, our aim was to investigate the prevalence of IRGM rs13361189, NKX2-3 rs10883365 and ECM1 rs13294 variants in large independent cohorts of Czech and Hungarian IBD patients. We also aimed to investigate a possible association between genotype and clinical phenotype, need for surgery, and response to medical therapy.

MATERIALS AND METHODS

Study population

One thousand seven hundred and seven unrelated IBD patients (CD: n = 810, age: 37.1 ± 12.6 years, duration: 10.7 ± 8.4 years and UC: n = 428, age: 43.7 ± 15.0 years, duration: 12.6 ± 9.9 years) and 469 healthy subjects (blood donors) from Hungary and the Czech Republic were investigated. The clinical data of the CD and UC patients are presented in Table 1. A detailed clinical phenotype was available in 789 CD and 422 UC patients.

The diagnosis was based on the Lennard-Jones criteria[24]. Age; age at onset; presence of extraintestinal manifestations [arthritis: peripheral and axial; ocular manifestations: conjunctivitis, uveitis and iridocyclitis; skin lesions: erythema nodosum and pyoderma gangrenosum; and hepatic manifestations: primary sclerosing cholangitis (PSC)]; frequency of flare-ups (frequent relapses: > 1 clinical relapse/year); therapeutic effectiveness (e.g. steroid and/or immunosuppressive use, steroid resistance); need for surgery (resections); the presence of familial IBD; smoking habits; and in CD, perianal involvement, were investigated by reviewing the medical charts by the physician and completing a questionnaire. The disease phenotype (age at onset, duration, location and behavior) was determined according to the Montreal classification[25].

The control group for mutation analysis consisted of 469 age- and sex-matched healthy blood donors (male/female: 251/218, age: 40.5 ± 11.5 years old). Control subjects did not have any gastrointestinal and/or liver diseases and were selected from consecutive blood donors in Budapest, Veszprem, Debrecen and Prague. The study protocol was approved by the Ethical and Science Committee of the Ministry of Health. Each patient was informed of the nature of the study and gave signed informed consent.

Genotyping and DNA isolation

Genomic DNA was isolated from whole blood according to the manufacturers' description with High Pure PCR Template preparation Kit (Roche, Budaors, Hungary).

Detection of IRGM (rs13361189,g.11386323T>C), ECM1 (rs13294, c.1243A>G, p.Ser415Gly, g.975342G>A) and NKX2-3 (rs1083365, g.20036290G>A) polymorphisms

Genotyping was performed using the LightCycler (Roche Diagnostics, Basel, Switzerland) allelic discrimination system. Amplification primers and hybridization probes were designed by the LightCycler Probe Design software (Roche Diagnostics). All oligonucleotides were synthesized by VBC Biotech (Vienna, Austria). The following amplification primers and hybridization probes were used for genotyping IRGM: IRGM-LCF: 5'-ATGGACAGTCAGTACCCTGCAC-3', IRGM-LCR: 5'-CTCTTTACCATTGTACTCCTTGTGCC-3', IRGM-ANC: 5'-LC Red 640-TGCTCAGCGGGTACAGTTTAGAAAGGGAA-Phosphate-3', IRGM-SENS: 5'-GAAAATCGGATGTATATTAGTAGACCC-Fluorescein-3'.

The amplification primers and hybridization probes used for genotyping of ECM1 were: ECM1-LCF: 5'-ACCCACCACCACTTGTGT-3', ECM1-LCR: 5'-TGCTTGGTGAGAACTCTTTGGTTT-3', ECM1-ANC: 5'-LC Red 640-TCAAGATGTCCCGGTCATAGTTGGGGTAAGGAG-Phosphate-3', ECM1-SENS: 5'-TGACTCGACCGATGTCAAT-Fluorescein-3'.

The amplification primers and hybridization probes used for genotyping of NKX2-3 were: NKX2-LCF:

Table 1 Clinical characteristics of patients with inflammatory bowel diseases

	CD (n = 810)	UC (n = 428)
Male/female (n)	434/376	202/226
Age (yr)	37.1 ± 12.6	43.7 ± 15.0
Age at presentation (yr)[1]	26.5 ± 10.6	31.3 ± 13.4
Duration (yr)[1]	10.7 ± 8.4	12.6 ± 9.9
Familial IBD[1]	11.8%	7.6%
Location in CD[1]		
L1	28.2%	
L2	18.9%	-
L3	51.2%	
L4 only	1.7%	
All L4	7.9%	
Maximum extent in UC[1]		
Proctitis	-	7.8%
Left-sided		52.3%
Extensive		39.9%
Behavior in CD[1]		
B1	34.9%	
B2	34.5%	-
B3	30.7%	
Perianal disease[1]	41.2%	-
Frequent relapse in CD/chronic continuous in UC[1]	39.9%	27.6%
Arthritis[1]	38.6%	27.1%
Cutaneous[1]	7.8%	5.9%
Occular[1]	7.7%	3.8%
PSC[1]	2.4%	3.8%
Steroid use/refractory[1]	81.1%/11.6%	66.2%/12.3%
Azathioprine use[1]	68.3%	29.3%
Anti-TNF use[1]	33.2%	10.4%
Surgery in CD/colectomy in UC[1]	50.9%	10.0%
Smoking habits[2]		
No	55.1%	71.6%
Yes	31.3%	12.4%
Previous	13.6%	16.0%

[1]Clinical phenotype available in 789 Crohn's disease (CD) and 422 ulcerative colitis (UC) patients; [2]Available in 691 CD and 388 UC patients. L1: Ileal; L2: Colon; L3: Ileocolonic; L4: Upper gastrointestinal; B1: Inflammatory; B2: Stenosing; B3: Penetrating. IBD: Inflammatory bowel disease; TNF: Tumor necrosis factor.

5'-CCGCATAAGACGTTACTTAAACATGT-3', NKX2-LCR: 5'-GCTATCTACTCGAAACTGTCT-GC-3', NKX2-ANC-2: 5'-TCTCCCCGGGGGTCAC-GTTG-Fluorescein-3', NKX2-SENS-2: 5'-LC Red 610-ACAAACACCTTCAAACCGTC-Phosphate-3'.

Polymerase chain reaction (PCR) was performed by LightCycler 480 real-time PCR System (Roche), in a reaction volume of 20 μL with 50 ng genomic DNA, 10 μL 2 × PCR Master Mix (Promega), and 5 pmol of the respective labeled oligonucleotides (sensor and anchor). For IRGM and NKX2-3, an asymmetric multiplex system was used for simultaneous amplification of the two fragments with 3:10 pmol forward:reverse (F:R) amplification oligonucleotides and 10:3 F:R amounts for IRGM and NKX2-3 SNPs, respectively. Similarly, for ECM1, an asymmetric PCR was applied with a 10:3 pmol F:R primer mix for amplification in a separate reaction. Cycling conditions were as follows: initial denaturation at 95℃ for 3 min, followed by 35 cycles of denaturation at 95℃ for 20 s, annealing at 55℃ for 30 s, and extension at 72℃ for 45 s, with a ramping rate of 4.4℃/s.

After amplification, a melting curve analysis was performed by cooling the samples to 40℃, followed by gradual heating to 80℃ with a ramp rate of 0.04℃/s. The decline in fluorescence was continuously monitored. Melting curves were converted to melting peaks with wild-type and variant alleles showing distinct melting points. For the IRGM and NKX2-3 multiplex PCR, color compensation was performed to compensate for the fluorescence crosstalk between detection channels. Genotype calling was carried out by two independent investigators.

Statistical methods

Variables were tested for normality using Shapiro Wilk's *W* test. A *t* test with separate variance estimates, χ^2 test and χ^2 test with Yates correction were used to evaluate differences between IBD patients and controls, as well as within subgroups of IBD patients. Logistic regression was used to compare genetic and clinical data and results are expressed as OR with 95% CI. $P < 0.05$ was considered as significant. For the statistical analysis, SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) was used. Statistical analysis was performed by Lakatos PL with the assistance of a statistician.

RESULTS

Association between NKX2-3, IRGM and ECM1 and disease susceptibility

All investigated polymorphisms were in Hardy-Weinberg equilibrium (P = 0.38-0.95). The success rate of the genotyping assays was 98%-99%. The genotype and allele frequencies are presented in Table 2. NKX2-3 rs10883365 variant allele was associated with increased risk for CD (P = 0.018 after Bonferroni correction, OR = 1.24, 95% CI = 1.06-1.48) and UC (P = 0.003 after Bonferroni correction, OR = 1.36, 95% CI = 1.13-1.63), whereas the variant IRGM allele increased the risk of CD (P = 0.04 after Bonferroni correction, OR = 1.36, 95% CI = 1.03-1.79). The association between NKX2-3 rs10883365 variant and IBD was also significant in the genotypic and dominant models. A similar trend was noted between IRGM and CD. In contrast, ECM1 rs13294 was not associated with either CD or UC. The combination of IRGM carrier/NKX2-3 homozygote genotype was significantly higher in CD compared to the controls (7.2% *vs* 2.1%, $P < 0.0001$ after Bonferroni correction, OR = 3.55, 95% CI = 1.80-7.01).

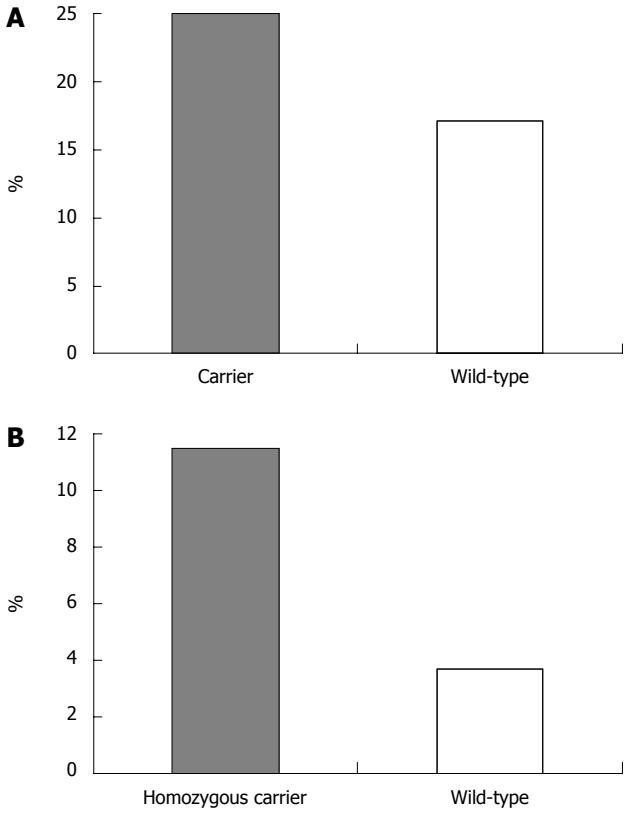

Figure 1 Association between immunity-related GTPase family, M and ECM1 and disease phenotype in patients with inflammatory bowel diseases. A: Association between the IRGM rs13361189 variant and disease location in Crohn's disease. P = 0.04, OR = 1.62, 95% CI: 1.07-2.44; B: Association between homozygous carriage of the ECM1 rs13294 variant allele and cutaneous manifestations in ulcerative colitis. P = 0.004, OR = 3.36, 95% CI: 1.48-7.63.

Role of NKX2-3, IRGM and ECM1 variants in predicting sex, familial disease, age at onset, disease location, phenotype or extraintestinal manifestations

In CD, presence of the variant IRGM allele was associated with colon-only location (in carriers: 25% *vs* wild-type: 17.1%, P = 0.04 after Bonferroni correction, OR = 1.62, 95% CI = 1.07-2.44, Figure 1A). In UC, homozygous carriage of the ECM1 variant allele was associated with cutaneous manifestations (11.5% in homozygotes *vs* 3.7% in patients with other genotypes, P = 0.004 after Bonferroni correction, OR = 3.36, 95% CI = 1.48-7.63, Figure 1B). No other significant associations were found in either CD or UC patients (data not shown).

Association between NKX2-3, IRGM and ECM1 and response to medical therapy or need for surgery

We also investigated the association between the NKX2-3, IRGM and ECM1 variants and the response to steroids,

Table 2 Association between NKX2-3, IRGM and ECM1 polymorphisms and disease susceptibility in patients with inflammatory bowel disease

	WT (%)	HET (%)	HOM (%)	*P* value	Carrier (%)	*P* value	OR (95%CI)	MAF	*P* value	OR (95%CI)
IRGM rs13361189										
All CD (*n* = 808)	636 (78.7)	156 (19.3)	16 (2.0)	0.060[1]	172 (21.3)	0.060[1]	-	188 (11.6)	0.029	1.36 (1.03-1.79)
Hungarian CD (*n* = 456)	355 (77.9)	89 (19.5)	12 (2.6)	NS[1,2]	101 (22.1)	NS[1,2]	-	113 (12.4)	NS[1,2]	-
Czech CD (*n* = 352)	281 (79.8)	67 (19.0)	4 (1.2)	NS[1,2]	71 (20.2)	NS[1,2]	-	75 (10.7)	NS[1,2]	-
All UC (*n* = 428)	351 (82.0)	76 (17.8)	1 (0.2)	NS[1,2]	77 (18.0)	NS[1,2]	-	78 (9.1)	NS[1,2]	-
Hungarian UC (*n* = 274)	221 (80.7)	52 (19.0)	1 (0.3)	NS[1,2]	53 (19.3)	NS[1,2]	-	54 (9.9)	NS[1,2]	-
Czech UC (*n* = 154)	130 (84.4)	24 (15.6)	0	NS[1,2]	24 (15.6)	NS[1,2]	-	24 (7.8)	NS[1,2]	-
All controls (*n* = 460)	382 (83.0)	75 (16.3)	3 (0.7)	-	78 (17.0)	-	-	81 (8.8)	-	-
Hungarian controls (*n* = 265)	217 (81.9)	46 (17.4)	2 (0.7)	-	48 (18.1)	-	-	50 (9.4)	-	-
Czech controls (*n* = 195)	165 (84.6)	29 (14.9)	1 (0.5)	-	30 (15.4)	-	-	31 (7.9)	-	-
NKX2 rs 10883365										
All CD (*n* = 810)	208 (25.7)	389 (48.1)	213 (26.2)	0.010[1]	602 (74.3)	NS[1,2]	-	815 (50.3)	0.009	1.24 (1.06-1.48)
Hungarian CD (*n* = 457)	115 (25.2)	220 (48.1)	122 (26.7)	NS[1,2]	342 (74.8)	NS[1,2]	-	464 (50.8)	NS[1,2]	-
Czech CD (*n* = 353)	93 (26.3)	169 (47.9)	91 (25.8)	0.070[1]	260 (73.7)	NS[1]	-	351 (49.7)	0.030[1]	1.31 (1.03-1.68)
All UC (*n* = 427)	90 (21.1)	226 (52.9)	111 (26.0)	0.004[1]	337 (78.9)	0.006[1]	1.52 (1.13-2.08)	448 (52.5)	0.001	1.36 (1.13-1.63)
Hungarian UC (*n* = 274)	56 (20.4)	152 (55.5)	66 (24.1)	NS[1,2]	218 (79.6)	0.070[1]		284 (51.8)	0.060	
Czech UC (*n* = 153)	34 (22.2)	74 (48.4)	45 (29.4)	0.017[1]	119 (77.8)	0.046[1]	1.63 (1.01-2.65)	164 (53.6)	0.005[1]	1.53 (1.14-2.07)
All controls (*n* = 469)	136 (29.0)	245 (52.2)	88 (18.8)	-	333 (71.0)	-	-	421 (44.9)	-	-
Hungarian controls (*n* = 271)	73 (26.9)	145 (53.5)	53 (19.6)	-	198 (73.1)	-	-	251 (46.3)	-	-
Czech controls (*n* = 198)	63 (31.8)	100 (50.5)	35 (17.7)	-	135 (68.2)	-	-	170 (42.9)	-	-
ECM1 rs13294										
All CD (*n* = 807)	139 (17.2)	392 (48.6)	276 (34.2)	NS[1,2]	688 (82.8)	NS[1,2]	-	944 (58.5)	NS[1,2]	-
Hungarian CD (*n* = 455)	89 (19.6)	228 (50.1)	138 (30.3)	NS[1,2]	366 (80.4)	NS[1,2]	-	504 (55.4)	NS[1,2]	-
Czech CD (*n* = 352)	50 (14.2)	164 (46.6)	138 (39.2)	NS[1,2]	302 (85.8)	NS[1,2]	-	440 (62.5)	NS[1,2]	-
All UC (*n* = 428)	77 (18.0)	225 (52.6)	126 (29.4)	NS[1,2]	351 (82.0)	NS[1,2]	-	477 (55.7)	NS[1,2]	-
Hungarian UC (*n* = 275)	44 (16.0)	156 (56.1)	75 (27.3)	NS[1,2]	231 (84.0)	NS[1,2]	-	306 (55.6)	NS[1,2]	-
Czech UC (*n* = 153)	33 (21.6)	69 (45.1)	51 (33.3)	NS[1,2]	120 (78.4)	NS[1,2]	-	171 (55.9)	NS[1,2]	-
All controls (*n* = 463)	90 (19.4)	222 (47.9)	151 (32.6)	-	373 (80.6)	-	-	524 (56.6)	-	-
Hungarian controls (*n* = 265)	56 (21.1)	124 (46.8)	85 (32.1)	-	209 (78.9)	-	-	294 (55.5)	-	-
Czech controls (*n* = 198)	34 (17.2)	98 (49.5)	66 (33.3)	-	164 (82.8)	-	-	230 (58.1)	-	-

[1]*P* value *vs* controls; [2]*P* value Crohn's disease (CD) *vs* ulcerative colitis (UC). WT: Wild-type; HET: Heterozygous carrier; HOM: Homozygous carrier; MAF: Minor allele frequency; NS: Not significant.

infliximab or azathioprine or the need for surgery in patients with CD. Two hundred and sixty-three unrelated CD patients were treated with anti-tumor necrosis factor (TNF): 259 received infliximab and four, adalimumab, two of whom were treated after secondary loss of response to infliximab therapy. Overall, there was no association between the presence of the above variants and short-term response (assessed at week 12) to infliximab induction therapy [5 mg/kg at weeks 0, 2 and 6; partial response: Crohn's disease activity index (CDAI) decreased by $\geqslant$ 70 points and/or $\geqslant$ 50% decrease in the number of draining fistulas; remission: CDAI < 150 or closure of all fistulas]; steroid use/resistance; azathioprine use; or need for surgery (data not shown). Similarly, no association was found between either of the variants and steroid use/resistance, azathioprine use or need for surgery in patients with UC (data not shown).

DISCUSSION

This is the first report on the prevalence of the IRGM rs13361189, NKX2-3 rs10883365 and ECM1 rs13294 variants, in large, independent IBD cohorts from Eastern Europe. The variant NKX2-3 allele conferred a risk in both UC and CD, whereas the IRGM rs13361189 variant allele was associated with increased risk for CD. In addition, the IRGM variant was associated with disease location in CD.

The genotype and allele frequencies for IRGM and NKX2-3 variants in CD, UC and controls reported in the present study were in line with most previous reports in Caucasian populations, whereas the frequency of the variant ECM1 rs13294 allele was approximately 10% higher in both IBD and controls, compared to that reported in previous studies[7,8,12,13,15,26].

We confirmed that the IRGM rs13361189 variant was associated with disease susceptibility in CD in Eastern European populations. The allelic OR of 1.36 (95% CI = 1.03-1.79) was in the range reported in Caucasian populations (OR = 1.38-1.56)[7,13] and in a recent meta-analysis (OR = 1.34)[10]. Similar genotype distributions were observed in both the Czech and Hungarian cohort, however, due to the low variant allele frequency, the difference became significant only in the combined analysis. A similar tendency (P = 0.06) was observed in the genotypic and dominant model. In contrast, in the present study we failed to replicate the weak association between IRGM and UC (rs13361189 P = 0.0069, pooled OR = 1.16; rs4958847 P = 0.014, pooled OR = 1.13) that was reported recently in a Spanish meta-analysis[10]. Of note, the present study was underpowered to detect such small

differences, however, there was even no trend for a difference between UC and controls.

In addition, in an Italian study[27], the rs4958847 polymorphism was associated with fistulizing behavior (P = 0.037, OR = 1.54, CI = 1.02-2.31) and perianal fistulas (P = 0.045, OR = 1.55, CI = 1.01-2.38) in a logistic regression analysis. This was later partially confirmed by the Leuven group. Henckaerts *et al*[28] have reported in a very elegant study a significant association between the IRGM rs4958847 variant, U7 gene desert rs12704036 T-allele, NOD2/CAR15 mutation, ileal involvement at diagnosis, male sex, and time to development of non-perianal fistulas in a Cox regression analysis. In a further study from New Zealand[29], rs13361189 variant increased the risk for ileal CD in 507 CD patients and 576 controls. The OR in ileal CD was 1.92 (95% CI = 1.27-2.96). Moreover, Peterson *et al*[30] have suggested an association between IRGM and pediatric disease onset (< 17 years) of CD in a North American cohort. However, only one of the two IRGM variants studied (rs13361189) was weakly associated with CD in their study (uncorrected P = 0.03). In the present study, we could not confirm the association between age at onset and the presence of the IRGM variant, in accordance with Canadian, Italian and Scottish results[29,31]. In contrast, we found an association between IRGM carriage and disease location in CD; colonic location was significantly more common in carriers of the variant allele (OR = 1.62, 95% CI = 1.07-2.44). Of note, however, the rs4958847 variant was not investigated in the present study.

The association between the rs10883365 variant of NKX2-3 gene and susceptibility of IBD was first reported in CD in Caucasian patients and in a Japanese study with an OR of 1.2-1.6[12-14]. In addition, using the UC panel and the expanded WTCCC control panel, Fisher *et al*[15] have reported a modest association ($P = 3.3 \times 10^{-4}$ and $P = 2.4 \times 10^{-6}$) between rs10883365 and UC. In the present study, we confirmed these findings; the rs10883365 variant was associated with UC and CD susceptibility in the allelic and genotypic models with an OR of 1.53 and 1.24, respectively. Based on our data, the association between NKX2-3 and IBD is stronger in UC compared to CD, at least in patients from Eastern Europe. In a recent Dutch study, the association between the rs10883365 variant of NKX2-3 and CD was linked to smoking status, with the risk being more pronounced in active and passive smokers[32]. In the present study, however, we did not find an association between NKX2-3 and smoking status, and similarly, no genotype-phenotype associations were found.

In addition, Weersma *et al*[13] have created genetic risk profiles by combining the presence of variant alleles in IL23R, ATG16L1, IRGM, NKX2-3, 1q24, 5p13, HERC2, CCNY, 10q21 and NOD2/CARD15, and the number of risk alleles was associated with gradually increased risk for CD. Similarly, in the present study, the combination of IRGM carrier/NKX2-3 homozygote genotype was associated with an increased risk for CD, with a much higher OR compared to either of the variant alleles alone.

Recently, an association between ECM1 rs3737240 and rs13294 variants and UC has been reported in a GWAS by Fisher *et al*[15], with an OR of 1.3-1.4 for the homozygous carriage of the variant allele. The association for the rs13294 variant has recently been confirmed in a Dutch study, with an OR of 1.24 in patients with UC[33]. In contrast, no association was found in CD[34], even though a different SNP (rs11205387) was investigated. Although the present study was powered to confirm a difference with an OR of 1.3-1.5 with adequate statistical power, we could not confirm an association between the ECM1 rs13294 variant and either UC or CD. The accidental association between homozygous carriage of the ECM1 variant allele and cutaneous manifestations (P = 0.002, OR = 3.36, 95% CI = 1.48-7.63, Figure 1B) requires further confirmation.

Theoretically, through influencing inflammatory responses, autophagy and epithelial-stromal interactions, polymorphisms in the above genes might be of potential importance in altering the efficacy of anti-inflammatory therapy and thereby the need for surgery. However, in the present study, none of the variant alleles was associated with the response to either steroid or infliximab therapy, or the need for surgery (resection in CD or colectomy in UC) in IBD.

In conclusion, NKX2-3 and IRGM are susceptibility loci for IBD in Eastern European patients. None of the variants investigated were associated with the need for surgery or efficacy of medical therapy. Further studies are needed to confirm the reported phenotype-genotype associations found in this study.

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COMMENTS

Background

Sequence variants in the autophagy gene immunity-related GTPase family M (IRGM) and NKX2-3 have been reported to contribute to Crohn's disease (CD) susceptibility, whereas ECM1 contributes to ulcerative colitis (UC) in genome-wide association scans in North America and Western Europe.

Research frontiers

There is a lack of data in Eastern European countries, therefore, our aim was to investigate the prevalence of IRGM rs13361189, NKX2-3 rs10883365 and ECM1 rs13294 variants. In addition, the possible association between genotype and clinical phenotype and the need for surgery is conflictive, and the association between the above genetic variants and response to medical therapy was not investigated.

Innovations and breakthroughs

In the present study, the authors showed in two well-characterized, independent

CD cohorts with strict clinical follow-up, that NKX2-3 rs10883365 variant allele was associated with increased susceptibility to CD and UC, whereas variant IRGM allele increased the risk for CD only. Our data suggest that the variant IRGM allele is associated with colon-only location, whereas in UC, the ECM1 variant was associated with cutaneous manifestations. None of the variants predicted resistance to steroids and azathioprine, efficacy of infliximab, or need for surgery.

Application

The new data presented from Eastern Europe might contribute to better understanding of genetic or environmental differences between populations and the association of those differences with disease susceptibility and phenotype.

Terminology

Vienna-Montreal classification: classification systems of disease phenotypes in CD. The classification assesses the age at presentation, disease location and disease behavior. The IRGM gene encodes an autophagy-inducing protein and plays an important role in host defense against intracellular pathogens. NKX2-3 is a member of the NKX family of homeodomain-containing transcription factors, which are implicated in many aspects of cell type specification and maintenance of differentiated tissue functions. ECM1 encodes extracellular matrix protein 1, a glycoprotein expressed in small and large intestine. Notably, ECM1 strongly activates nuclear-κB signaling, a key immune regulator.

Peer review

The need to extend studies performed in the other parts of the world to Eastern Europe is valid, and provides a point for understanding subtle genetic or environmental differences between populations and the association of those differences with disease.

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BRIEF ARTICLE

Clinical analysis of high serum IgE in autoimmune pancreatitis

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Abstract

AIM: To clarify the clinical significance of high serum IgE in autoimmune pancreatitis (AIP).

METHODS: Forty-two AIP patients, whose IgE was measured before steroid treatment, were analyzed. To evaluate the relationship between IgE levels and the disease activity of AIP, we examined (1) Frequency of high IgE (> 170 IU/mL) and concomitant allergic diseases requiring treatment; (2) Correlations between IgG, IgG4, and IgE; (3) Relationship between the presence of extrapancreatic lesions and IgE; (4) Relationship between clinical relapse and IgE in patients treated with steroids, and (5) Transition of IgE before and after steroid treatment.

RESULTS: IgE was elevated in 36/42 (86%) patients. Concomitant allergic disease was observed in seven patients (allergic rhinitis in three, bronchial asthma in three, and urticaria in one). There were no significant correlations between IgG, IgG4, and IgE (r = -0.168 for IgG, and r = -0.188 for IgG4). There was no significant difference in IgE in the patients with and without extrapancreatic lesions (526 ± 531 IU/mL *vs* 819 ± 768 IU/mL, P = 0.163), with and without clinical relapse (457 ± 346 IU/mL *vs* 784 ± 786 IU/mL, P = 0.374). There was no significant difference in IgE between before and after steroid treatment (723 ± 744 IU/mL *vs* 673 ± 660 IU/mL, P = 0.633).

CONCLUSION: Although IgE does not necessarily reflect the disease activity, IgE might be useful for the diagnosis of AIP in an inactive stage.

Key words: IgE; IgG4; IgG; Autoimmune pancreatitis

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INTRODUCTION

Autoimmune pancreatitis (AIP) is a unique, benign pan-

creatic disease characterized by irregular narrowing of the pancreatic duct, swelling of the pancreas, lymphoplasmacytic infiltration and fibrosis, and favorable response to steroid therapy[1-8]. Serologically, elevation of IgG and IgG4 is the most remarkable characteristic in this disease[9-12]. A recent study showed that IgM and IgA were decreased in AIP[13]. There has been no detailed clinical analysis of IgE, although some clinicians have noted elevated serum IgE in AIP or IgG4-related diseases[14-17].

In most allergic diseases, total serum IgE levels do not reflect disease activity; however, in allergic bronchopulmonary aspergillosis, it is reported that IgE is a useful marker for therapeutic monitoring[18-21]. The expression of T-helper type 2 (Th2) cytokines [interleukin (IL)-4, IL-5, and IL-13] are upregulated in the affected tissues of AIP[22]. Both IgG4 and IgE production are dependent on help by Th2; therefore, all IgG4-inducing antigens are also efficient IgE inducers[23]. As IgG4 reflects the disease activity of AIP[12], it is reasonable to expect that IgE is also related to the disease activity of AIP and could become a clinically useful marker. Thus, we decided to clarify whether IgE is related to the disease activity of AIP from various viewpoints.

MATERIALS AND METHODS

Patients

Between 1997 and 2009, 67 patients were diagnosed as having AIP at the University of Tokyo hospital and affiliated hospitals. All the patients fulfilled the diagnostic criteria of AIP proposed by the Mayo Clinic[3] or the revised criteria by the Japan Pancreas Society[4]. Serum IgE was measured in 48 patients before steroid treatment. As the method of measurement was different in six patients, these patients were excluded. Thus, 42 patients whose IgE level was measured by the same method before steroid treatment were enrolled in this study. Of the 42 patients, 33 were men and nine were women. The mean age of onset was 65 years old. Thirty-seven patients received steroid treatment. Prednisolone at an initial dose of 30-40 mg/d was administered for 2-4 wk in most cases. It was then tapered by 5 mg every 2-6 wk until 10 mg/d, and 2.5-7.5 mg/d was continued as maintenance therapy in principle.

This retrospective study was approved by the review board of our institute.

Methods

Serum IgE was measured by fluorescence enzyme immunoassay. To evaluate the relationship between IgE levels and disease activity, we examined (1) frequency of high IgE (> 170 IU/mL) and concomitant allergic diseases requiring treatment; (2) correlations among IgG, IgG4, and IgE; (3) relationship between the presence of extrapancreatic lesions and IgE; (4) relationship between clinical relapse and IgE in patients treated with steroids; and (5) transition of IgE before and after steroid treatment.

With regard to allergic diseases, only diseases that required treatment during follow-up were counted. There are many extrapancreatic lesions in AIP; however, in this study, only representative and definite lesions, including sclerosing cholangitis[7,8,24], retroperitoneal fibrosis[8,25], sclerosing sialadenitis[17], interstitial pneumonia[26], and tubulointerstitial nephritis[27] were counted. With regard to the number of extrapancreatic lesions, we used the number of extrapancreatic lesions that were observed when IgE was measured. We do not regard intrapancreatic biliary stricture as an extrapancreatic lesion, because it is influenced by pancreatic edema[24]. We defined "clinical relapse" as AIP-related symptomatic unfavorable events; i.e. obstructive jaundice from distal bile duct stenosis due to exacerbated pancreatitis with pancreatic swelling, increased levels of biliary enzymes caused by sclerosing cholangitis (in which extrapancreatic biliary strictures were confirmed on imaging findings), retroperitoneal fibrosis, interstitial pneumonia, and interstitial nephritis (for which simple observation seemed very inadequate and remission induction therapy was introduced). Concerning clinical relapses and IgE, we analyzed patients whose follow-up after the initiation of steroid treatment was more than 6 mo. With regard to the transition of IgE, IgE measured before steroid treatment and during maintenance steroid treatment (2.5-7.5 mg prednisolone/d) were compared.

Statistical analysis

Categorical variables were compared by the χ^2 or Fisher exact test, where appropriate. Continuous variables were reported as mean ± SD and compared by the Student *t* test, Welch *t* test, or paired *t* test, where appropriate. A *P* value of < 0.05 was considered statistically significant. Statistical analyses were performed by the statistical software JMP 7.0.1 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Frequency of high IgE and concomitant allergic diseases

The clinical profiles of 42 patients with AIP are summarized in Table 1. Serum IgE was elevated in 36/42 (86%). The average value of IgE was 679 ± 675 IU/mL (range, 67-3000 IU/mL). No patient had concomitant parasitosis. Concomitant allergic diseases were observed in seven patients, comprising allergic rhinitis in three, bronchial asthma in three, and urticaria in one. There was no significant difference between the average IgE values of these seven patients and those of the other 35 patients (970 ± 775 IU/mL *vs* 621 ± 650 IU/mL, $P = 0.216$). The frequency of high IgE was 100% (7/7) in these patients, and 63% (29/35) in the others; however, this difference was not statistically significant ($P = 0.567$).

Correlations between IgG, IgG4, and IgE

The values of IgG and IgG4, which were measured at the same time as IgE before steroid treatment, were used in this analysis. Elevation of IgG and IgG4 were observed in 20 (47%) and 39 (93%) patients, respectively. The correlation coefficient of IgG and IgE was -0.168 (not significant, $P = 0.290$). The correlation coefficient of IgG4 and IgE was -0.188 (not significant, $P = 0.235$). The correla-

Table 1 Clinical profiles of 42 patients with autoimmune pancreatitis

Patient	Sex	Age	IgE (< 171 U/mL)	IgG (870-1800 mg/dL)	IgG4 (< 135 mg/dL)	Total bilirubin (0.3-1.3 mg/dL)	Concomitant allergic diseases	Extrapancreatic lesion associated with AIP
1	F	58	670	2542	592	0.6	AR	-
2	M	63	650	2055	691	3.0	AR	-
3	F	53	500	1527	143	7.9	AR	SA
4	M	64	490	2457	670	0.5	BA	-
5	M	56	1800	1712	436	0.5	BA	-
6	F	43	340	1036	223	0.9	BA	-
7	M	74	2339	1481	98	0.9	Urticaria	RF
8	F	70	480	2190	133	5.8	-	-
9	M	57	120	3793	1420	10.6	-	RF
10	M	55	480	1419	320	1.7	-	-
11	M	61	270	1878	410	0.4	-	SC
12	M	66	1200	1620	310	0.5	-	SA
13	M	79	170	1585	420	3.3	-	SC
14	M	73	290	1647	360	5.4	-	-
15	M	79	410	1404	554	0.7	-	-
16	M	76	1000	1728	65	14.1	-	SC
17	F	72	650	2384	1400	1.6	-	SA
18	F	65	190	1511	374	0.6	-	-
19	M	68	290	1656	253	2.0	-	SC
20	M	61	940	1448	578	1.6	-	-
21	F	61	640	2177	354	0.4	-	-
22	M	58	2289	1973	481	6.9	-	-
23	M	71	1915	2318	470	0.6	-	-
24	M	61	69	2215	974	0.5	-	SA
25	M	65	91	3032	1260	5.4	-	SC, RF
26	M	64	330	1730	361	0.4	-	SC
27	M	66	1330	1849	270	4.7	-	-
28	F	64	328	2898	456	12.8	-	SC
29	M	69	440	1875	270	8.7	-	-
30	M	73	70.3	2395	393	0.4	-	RF
31	M	67	660	1683	230	0.6	-	-
32	M	72	3000	1579	455	0.5	-	-
33	M	61	467	1301	331	2.9	-	-
34	M	40	320	1996	650	1.0	-	RF
35	M	62	480	1368	236	0.5	-	SC, SA
36	M	71	267	1827	458	1.3	-	-
37	M	59	625	1876	139	0.5	-	RF
38	M	73	302	2834	1800	0.5	-	SA
39	F	76	426	1458	543	0.9	-	RF
40	M	76	943	1840	431	0.7	-	SC
41	M	59	67	1338	140	0.7	-	-
42	M	64	198	1709	232	10.2	-	-

AIP: Autoimune pancreatitis; AR: Allergic rhinitis; BA: Bronchial asthma; SA: Sialadenitis; RF: Retroperitoneal fibrosis; SC: Sclerosing cholangitis.

Table 2 Correlations between IgG, IgG4, and IgE

	Correlation coefficient	*P* value
IgG and IgE	-0.168	0.290
IgG4 and IgE	-0.188	0.235
IgG and IgG4	0.698	< 0.0001

tion coefficient of IgG and IgG4 was 0.698, which was significant ($P < 0.0001$) (Table 2).

Relationship between the presence of extrapancreatic lesions and IgE

Extrapancreatic lesions were observed in 20 patients (48%). Two patients had two lesions, and 18 patients had one lesion. Sclerosing cholangitis, retroperitoneal fibrosis, and sclerosing sialadenitis, were observed in nine, seven, and six patients, respectively. The IgE levels of the patients with and without extrapancreatic lesions were compared, and the same analysis was performed for IgG and IgG4. The results are shown in Table 3. IgG tended to be related to the presence of extrapancreatic lesions, although statistical significance was not attained ($P = 0.093$). No such tendency existed for IgE.

Table 3 Comparison of IgE, IgG, and IgG4 between patients with and without extrapancreatic lesions

	Patients with extrapancreatic lesions (n = 20)	Patients without extrapancreatic lesions (n = 22)	*P* value
IgE (IU/mL)	526 ± 531	819 ± 768	0.163
IgG (mg/dL)	2065 ± 644	1775 ± 396	0.093
IgG4 (mg/dL)	588 ± 505	392 ± 161	0.110

Table 4 Comparison of IgE, IgG, and IgG4 between patients with and without clinical relapses

	Patients with clinical relapses (n = 5)	Patients without clinical relapses (n = 28)	P value
IgE (IU/mL)	457 ± 346	784 ± 786	0.374
IgG (mg/dL)	1898 ± 424	1915 ± 579	0.953
IgG4 (mg/dL)	266 ± 157	558 ± 429	0.148

Table 5 Transition of IgE, IgG, and IgG4 before and after steroid treatment

	Before	After	P value
IgE (IU/mL) (n = 29)	723 ± 744	673 ± 660	0.633
Proportion of high IgE	26/29	23/29	0.470
IgG (mg/dL) (n = 30)	1891 ± 566	1155 ± 315	< 0.0001
Proportion of high IgG	14/30	1/30	0.0002
IgG4 (mg/dL) (n = 28)	557 ± 429	229 ± 112	0.0002
Proportion of high IgG4	27/28	20/28	0.0248

Normal range, IgE: < 171 IU/mL, IgG: 870-1800 mg/dL, IgG: < 135 mg/dL.

Relationship between clinical relapse and IgE in the patients treated with steroids

There were 33 patients whose follow-up period was more than 6 mo. The mean follow-up period was 52 mo (range, 8-141 mo). Clinical relapse was observed in five patients. The style of clinical relapse was pancreatitis in two, interstitial pneumonia in two, and sclerosing cholangitis in one. Their relapses occurred 16 mo after the initiation of steroid therapy on average (range, 3-26 mo). The mean follow-up period was the same between the patients with and without clinical relapse (53.0 mo *vs* 51.8 mo, $P = 0.929$). IgE levels of the patients with and without clinical relapse were compared, and the same analysis was performed for IgG and IgG4. The results are shown in Table 4. Neither IgE, IgG nor IgG4, were related to later clinical relapses.

Transition of IgE before and after steroid treatment

IgE measured before steroid treatment and during maintenance therapy could be compared in 29 patients (Figure 1). IgE increased in 10, and decreased in 18. There was no significant difference in IgE between before and after steroid treatment (723 ± 744 IU/mL *vs* 673 ± 660 IU/mL, $P = 0.633$). Abnormally high IgE (> 170 IU/mL) was observed in 90% (26/29) before steroid treatment, and in 79% (23/29) after steroid treatment ($P = 0.470$). By contrast, IgG and IgG4 did show significant differences before and after steroid treatment (Table 5).

DISCUSSION

High IgE in AIP has been frequently documented[14-17], but its frequency and clinical significance were unknown. Kamisawa *et al*[14] reported that elevation of IgE was observed in 34% (12/35) of patients, and that all the patients with high IgE had present and/or past histories of allergic diseases, although none of the patients with normal IgE had such histories. In the present study, the

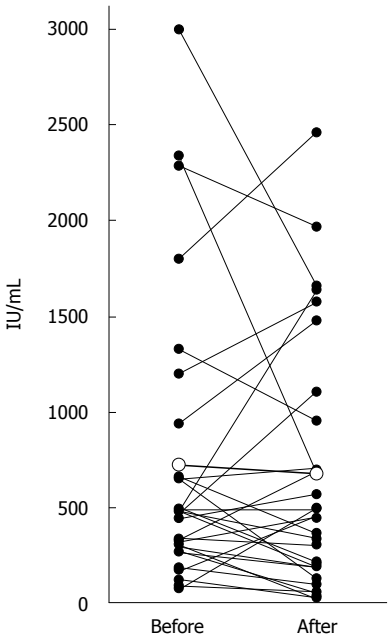

Figure 1 IgE levels measured before steroid treatment (Before) and during maintenance therapy (After) were compared in 29 patients. White circles show average values.

frequency of high IgE was surprisingly high at 86% (36/42), which might be equal to frequency of high IgG4 (73.3%-94.3%)[12]. On the other hand, unlike the previous report, there seemed no definite relationship between IgE and allergic diseases. It seemed unreasonable to count allergic diseases that occurred decades ago. In addition, it is difficult to accurately judge past histories of mild allergic diseases. Thus, we included only concomitant allergic diseases. For reference, there were at least eight patients who had past histories of allergic diseases, but no concomitant ones. Comparison between patients with (n = 15) and without (n = 27) present and/or past histories of allergic diseases showed no significant difference in mean IgE values (654 ± 605 IU/mL *vs* 693 ± 721 IU/mL, $P = 0.860$) and frequency of high IgE (93% *vs* 81%, $P = 0.395$); therefore, the presence of past allergic disease did not affect the results.

From the results shown in Table 3, IgE levels appear to be unrelated to disease activity from the viewpoint of extrapancreatic lesions. On the contrary, it is possible that high IgE is associated with lower disease activity, when considering the higher IgE levels in the group without extrapancreatic lesions, and the negative correlation coefficient of IgG (IgG4) and IgE. It is difficult to analyze the results shown in Table 4 because of the small number of patients with clinical relapses. Nevertheless, it is likely that high IgE is not a risk factor for later clinical relapses, especially considering the higher IgE levels in the group without clinical relapses. IgG4 seems a little high in the group without clinical relapses (Table 4), which is similar to previous reports[8,28].

It was of great interest whether IgE could become a useful marker for therapeutic monitoring in AIP, like IgG and IgG4. From the results shown in Table 5, we

cannot help but conclude that IgE is not a useful marker. However, this phenomenon is not strange in other allergic diseases. For example, Gunnar *et al*[18] reported that steroid treatment did not alter IgE levels in patients with atopic dermatitis. Kumar *et al*[19] showed that changes in serum IgE are not related to severity of asthma or allergic rhinitis. Exceptionally, in allergic bronchopulmonary aspergillosis, it is reported that the response of IgE (35% or more reduction) to steroid treatment is a sensitive marker in the management[20].

Although IgE does not seem to reflect disease activity, we speculate that this feature might be useful for the diagnosis of inactive AIP. Indeed, three patients in the present series showed low IgG4 (65, 98, and 133 mg/dL) at the diagnosis, but all of them showed high IgE (1000, 2339 and 480 IU/mL). When patients with a past history suggestive of AIP, such as voluntarily improved jaundice, do not show high IgG and IgG4, IgE should be measured. If IgE is also low, the possibility that their diagnosis is AIP will be low. If IgE is high, it might indicate AIP in an inactive stage.

In summary, the elevation of serum IgE is very frequent in AIP. It is also observed even in patients without other allergic diseases. IgE might not reflect the disease activity; however, it might be useful for the diagnosis of AIP in an inactive stage.

COMMENTS

Background

It is known that elevation of serum IgE is frequently observed in autoimmune pancreatitis (AIP). However, its clinical significance has not yet been clarified.

Research frontiers

This study demonstrated the frequency of high IgE in AIP, and investigated whether IgE is related to the presence of extrapancreatic lesions and later clinical relapses. In addition, the transition of IgE before and after steroid treatment was investigated to confirm whether IgE can become a marker for therapeutic monitoring.

Innovations and breakthroughs

This study confirmed the high frequency of elevated serum IgE in AIP, although IgE does not seem to be related to the disease activity.

Applications

Measuring IgE might be useful for the diagnosis of AIP especially in an inactive stage.

Peer review

These data about IgE were not positive, which means that IgE is not considered as a useful marker for AIP. However, the author revealed that many (86%) AIP patients have IgE elevation and some AIP patients with low IgG4 have a high level of IgE. IgE should be considered as one of the supportive parameters for diagnosis of AIP and the authors succeeded in clarifying that.

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BRIEF ARTICLE

Segmental gastrectomy with radical lymph node dissection for early gastric cancer

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Abstract

AIM: To describe a new surgical technique and evaluate the early results of segmental gastrectomy (SG) with modified D2 lymph node (LN) dissection for early gastric cancer (EGC).

METHODS: Fourteen patients with EGC underwent SG with modified D2 dissection from 2006 to 2008. Their operative results and postoperative courses were compared with those of 17 patients who had distal gastrectomy (DG) for EGC during the same period.

RESULTS: Operating time, blood loss, and hospital stay were similar between the 2 groups. Postoperative complications developed significantly more frequently in the DG group than in the SG group. Mean number of dissected LNs per each station in the SG group was comparable with that in the DG group. Postoperative recovery of body weight was significantly better in the SG group than in the DG group. The incidence of reflux esophagitis and gastritis after surgery was less frequent in the SG group than in the DG group.

CONCLUSION: SG with modified D2 LN dissection may be a new function-preserving gastrectomy that is feasible for treatment of EGC with possible LN involvement.

Key words: Gastrectomy; Early diagnosis; Gastric cancer; Lymph node; Metastasis; Gastrointestinal surgical procedures

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INTRODUCTION

Less invasive surgery for early gastric cancer (EGC) has been more commonly employed in Japan[1-4]. Segmental gastrectomy (SG), which was originally applied for peptic ulcers, is one type of limited gastrectomy available for treating EGC[5]. SG for EGC is generally accompanied only by dissection of the lymph nodes (LNs) adjacent to tumors, although only a few studies regarding the application of SG in EGC have been reported[1-4]. To establish SG with adjacent LN dissection as a valid option for surgical treatment of EGC, it is necessary to demonstrate intraoperatively that there is no lymph node metastasis (N0). However, at present, it is difficult to determine the status of LN metastasis intraoperatively, even using the sentinel node (SN) navigation method[6-8].

The superiority of limited gastrectomy over conven-

tional D2 gastrectomy for EGC in terms of postoperative quality of life seems apparent, as previously reported[1,4,9,10]. In 1995, Sawai *et al*[11] developed pylorus-preserving gastrectomy (PPG) with D2 dissection by preservation of the infrapyloric artery as a function-preserving gastrectomy. Although this technique is considered to be applicable to submucosal or undifferentiated gastric cancer, there remains a risk of adverse events such as postoperative gastric stasis and reduced capacity of the remnant stomach.

We developed SG with modified D2 dissection and have applied it to EGC that do not meet endoscopic treatment criteria. We report the technique here and evaluate the early results of SG with modified D2 dissection for EGC.

MATERIALS AND METHODS

Between April 2006 and November 2008, 35 patients with EGC in the middle or lower third of the stomach which did not meet endoscopic treatment criteria underwent gastrectomy at our institution: 17 patients who underwent SG and 18 patients who underwent conventional distal gastrectomy (DG) with D2 dissection. The criteria for endoscopic treatment is EGC with differentiated histology, invasion limited to the mucosal layer, a diameter smaller than 2 cm, and no ulcer findings. In this study, SG was distinguished from PPG by defining that the remaining pyloric cuff needed to be more than 4 cm in SG. When the distance from the distal edge of the resection to the pylorus was < 4 cm, DG was performed instead of PPG.

A total of 14 patients received SG with modified D2 dissection. The remaining 3 patients who were excluded from this study received SG only with adjacent LN dissection because of intraoperative determination of apparent mucosal cancer with no LN metastasis.

In 3 out of 18 patients in the DG group, SG was converted to DG based on intraoperative findings including suspected T2 tumor or positive LN metastasis.

Patients were followed every 3 mo after surgery. The follow-ups also included an endoscopy and computed tomography 1 year after surgery. The operative and postoperative results were compared between the SG and DG groups.

Operative procedures

To make it possible to locate the tumor intraoperatively, proximal and distal margins of the tumor were marked by endoscopic clipping before surgery. After laparotomy, we explored the abdominal cavity and determined if the preoperative diagnosis was correct. If a T2 or T3 tumor was suspected or LN metastases were apparent, DG or total gastrectomy (TG) with D2 dissection was performed. Swollen and/or hard palpable LNs located adjacent to the tumor were excised and immediately examined by frozen section analysis. Infrapyloric LNs (station 6) and those along the common hepatic artery (CHA; station 8a) were also routinely subjected to frozen section analysis. DG or TG with D2 dissection was performed if LN metastasis was proved by frozen section analysis, however, if T1N0 was confirmed SG with modified D2 dissection was carried out.

The resection line of the stomach was made 2 cm and 5 cm away from the tumor margin with differentiated and undifferentiated histology, respectively. The greater omentum was preserved, and the gastrocolic ligament corresponding to the resection area of the stomach was dissected, after separation, at least 3 cm away from the gastroepiploic vessels. For dissection of LNs located in station 6 and along the greater curvature (station 4d), right gastroepiploic vessels were skeletonized, allowing excision of adjacent LNs, and divided at the distal resection line of the stomach (Figure 1A). The suprapyloric LNs (station 5) were dissected in the same manner. The right gastric artery was skeletonized and divided distally to the second or third branch (Figure 1B). The left halves of LNs along the proper hepatic artery (station 12a) were removed, and the hepatic and pyloric branches of vagus nerves were preserved. Right paracardial LNs (station 1), LNs along the lesser curvature (station 3), and the left gastric artery (LGA) (station 7) were dissected by dividing the LGA at its origin. The celiac branch of the vagus nerve was also dissected. Dissection of the upper portion of station 1 LNs was occasionally omitted in order to preserve the origin of the hepatic branch of the vagus nerve and the anterior branch of the Latarjet nerve. LNs along the CHA (station 8a, 8p), around the celiac axis (station 9), and along the proximal splenic artery (station 11p) were routinely dissected. LNs along the left gastroepiploic artery (LGEA) (station 4sb) were dissected by dividing the last few branches of the LGEA. After transection of the stomach, the surgical margins were subjected to intraoperative histological examination by frozen section analysis. Reconstruction was performed using hand-sewn end-to-end anastomosis (Figure 1C).

Statistical analysis

Statistical analysis was performed using either the χ^2 test or Student's *t*-test. A *P*-value < 0.05 was considered significant.

RESULTS

There were no differences between the 2 groups in terms of sex, age, and tumor characteristics, with the exception of tumor location (Table 1). In 1 patient of the DG group and 2 patients of the SG group, LN metastasis was proven to be positive.

Operative data are shown in Table 2. Operating time, blood loss, and hospital stay were similar between the two groups. However, postoperative complications developed significantly more frequently in the DG group than in the SG group. There were no instances of anastomotic leakage, hemorrhage, gastric stasis, wound infection or operative death in either group.

The mean number of dissected LNs per station was comparable between the 2 groups (Table 3).

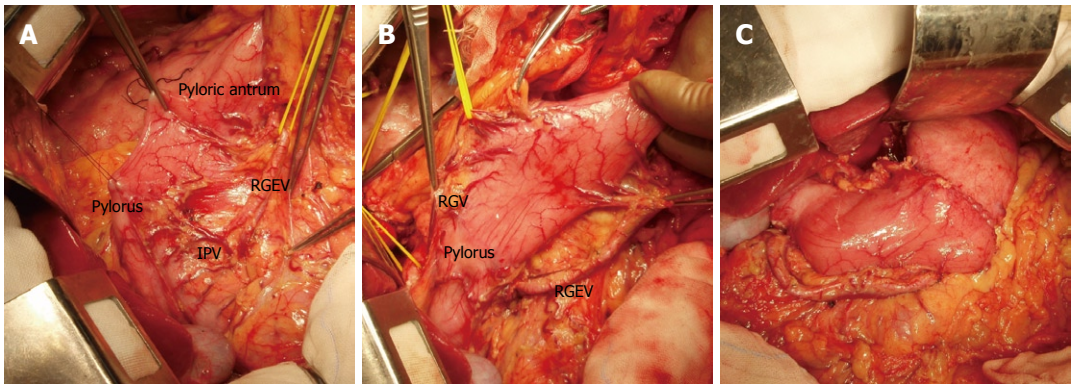

Figure 1 Photographs during surgery. A: The right gastroepiploic vessels were skeletonized allowing excision of adjacent lymph nodes (LNs) (stations 6 and 4 d). The surrounding nerve plexuses were preserved; B: For dissection of the suprapyloric LNs (station 5), the right gastric artery was skeletonized and divided distally to the 3rd branch; C: Reconstruction was performed using hand-sewn end-to-end anastomosis. RGEV: Right gastroepiploic vessels; IPV: Infrapyloric vessels; RGV: Right gastric vessels.

Table 1 Comparison of clinicopathological characteristics between the 2 groups

	SG (*n* = 14)	DG (*n* = 18)	*P*
Age (yr, mean ± SD)	63.9 ± 9.6	60.0 ± 12.9	0.204
Sex (M/F)	9/5	7/11	0.154
Location of tumor			0.002
Upper	0	0	
Middle	13	7	
Lower third of the stomach	1	11	
Depth of invasion			0.589
Mucosa	4	8	
Submucosa	8	9	
Muscularis propria	1	1	
Subserosa	1	0	
Histological type			
Differentiated	10	11	0.358
Undifferentiated	4	7	
Lymph node metastasis			0.401
No	12	17	
Yes	2	1	

SG: Segmental gastrectomy; DG: Distal gastrectomy.

Table 2 Operative results (mean ± SD)

	SG (*n* = 14)	DG (*n* = 18)	*P*
Operating time (min)	273 ± 65	262 ± 46	0.589
Blood loss (mL)	206 ± 124	179 ± 101	0.511
Hospital stay (d)	14.9 ± 2.6	16.7 ± 5.0	0.227
Complications	0	5	0.032
Wound infection	0	0	
Anastomotic leakage	0	0	
Hemorrhage	0	0	
Cholecystitis	0	2	
Pancreatitis	0	1	
Pancreatic fistula	0	1	
Adhesive ileus	0	1	
Operative death	0	0	1.000

SG: Segmental gastrectomy; DG: Distal gastrectomy.

Table 3 Numbers of dissected lymph nodes according to each station (mean ± SD)

Lymph node station	SG (*n* = 14)	DG (*n* = 18)	*P*
Right paracardial (1)	2.0 ± 1.3	3.1 ± 1.2	0.068
Left paracardial (2)	1.1 ± 1.5	0.7 ± 1.6	0.462
Along the lesser curvature (3)	8.7 ± 4.7	10.8 ± 7.3	0.362
Along the LGEA (4sb)	1.2 ± 2.1	1.4 ± 1.9	0.754
Along the RGEA (4d)	9.2 ± 4.9	11.7 ± 4.9	0.155
Suprapyloric (5)	0.3 ± 0.6	0.6 ± 0.9	0.286
Infrapyloric (6)	3.7 ± 3.7	5.6 ± 2.6	0.118
Along the LGA (7)	3.7 ± 2.4	4.3 ± 2.7	0.473
Along the CHA (8)	2.7 ± 1.2	3.3 ± 1.5	0.232
Along the celiac axis (9)	2.8 ± 1.6	3.3 ± 2.0	0.442
Along the proximal SA (11p)	1.5 ± 1.5	2.0 ± 1.7	0.412
Along the PHA (12a)	0.5 ± 1.0	0.2 ± 0.5	0.246
Along the SMV (14v)	-	1.2 ± 2.1	-

SG: Segmental gastrectomy; DG: Distal gastrectomy; LGEA: Left gastroepiploic artery; RGEA: Right gastroepiploic artery; LGA: Left gastric artery; CHA: Common hepatic artery; SA: Splenic artery; PHA: Proper hepatic artery; SMV: Superior mesenteric vein.

Table 4 Postoperative course (mean ± SD)

	SG (*n* = 14)	DG (*n* = 18)	*P*
Gastric stasis	0	0	1.000
Dumping syndrome	1	1	0.854
Body weight change ratio (%)	96.8 ± 6.1	90.4 ± 7.0	0.012
Laboratory data			
Lymphocytes (/mm^3)	2007 ± 437	1977 ± 646	0.885
Total protein (g/dL)	6.8 ± 0.3	7.0 ± 0.3	0.156
Total cholesterol (mg/dL)	215 ± 34	192 ± 32	0.063
Endoscopic examination			
Reflux gastritis	1	5	0.138
Reflux esophagitis	0	4	0.059

SG: Segmental gastrectomy; DG: Distal gastrectomy.

The postoperative course is summarized in Table 4. Postoperative recovery of body weight was significantly better in the SG group than in the DG group. The incidence of reflux esophagitis and gastritis after surgery was less frequent in the SG group than in the DG group.

No recurrence or death was observed in either group during a median follow-up period of 32.8 mo. One patient in the SG group developed colon cancer during the

follow-up period and subsequently underwent curative resection.

DISCUSSION

SG has not been previously used to treat EGC, since it was first reported by Wangensteen *et al*[5] as an operation for peptic ulcer. In 1999, Ohwada *et al*[2] modified the procedure and reported its use for treatment of EGC of the middle third of the stomach. However, previous studies concerning the use of SG for the treatment of EGC are limited, primarily due to difficulty in diagnosing LN metastasis accurately during surgery, and it being performed with very limited LN dissection. Deterioration of the radicality caused by omitting or limiting LN dissection should be avoided, because EGC can be treated with conventional D2 gastrectomy with excellent prognosis. Therefore, SG with limited LN dissection is not recommended unless T1N0 is confirmed.

On the other hand, a method for accurate intraoperative diagnosis of LN metastasis has yet to be established. Recently, SN biopsy has been used to assist intraoperative diagnosis of LN metastasis during surgical treatment of EGC. The SN concept seems feasible for treatment of EGC as described previously, however, several important issues including optimal tracer, method of injection, and false negative cases, still need to be resolved[6-8].

Our modified D2 dissection was considered to be comparable with conventional D2 dissection, because dissected stations in our SG corresponded with those in conventional D2 dissection. The mean number of dissected LNs of station 1 tended to be smaller in the SG group than in the DG group. This will be because dissection of the upper half of station 1 was occasionally omitted in order to preserve the anterior branches of the vagus nerve along the lesser curvature of the proximal remnant stomach, which may help maintain postoperative motility of this portion. The influence of limited dissection of station 1 on the oncological outcome is unclear, however, tumor recurrence resulting from incomplete dissection of station 1 has not been seen so far. Even in 3 patients whose LN metastasis was positive in the DG or SG group, no recurrence has been observed. In 1 of 2 patients whose LN metastasis was positive in the SG group, positive LN metastasis was proved after surgery in station 7 which belongs to the second tier. In the other 2 patients, LN metastasis was positive in station 3. These results may support the quality and effectiveness of our modified D2 dissection and the impact of D2 dissection.

In our procedures, 2-5 LNs were subjected to frozen section analysis. Station 8a was routinely examined by frozen section analysis. The frequency of LN metastasis in station 8a is relatively high even in EGC, and stations 7, 8a, and 11p are sometimes SN stations although they belong to the second tier[6,8,12-14]. Once the tumor advances to stage T2 or T3, lymphatic vessels can be obstructed by cancerous invasion, and metastasis in distant LNs can occur even in the absence of involvement of LNs adjacent to tumors. In the case where station 7 LN was proved positive after SG, intraoperative frozen section analysis detected no metastasis of stations 3 and 8a. Therefore, it is not recommended to limit LN dissection even if frozen section analysis reveals no LN involvement. Frozen section analysis with LN sampling was performed by us in order to rule out patients with positive LN metastasis rather than to confirm N0.

Unexpectedly, postoperative gastric stasis has not been observed in our patient population thus far. The precise mechanisms of development of gastric stasis after limited gastrectomy such as PPG remain unclear[15-18]. The main causes of gastric stasis are generally thought to include damage to the vagus nerve, insufficient blood supply to the pyloric region, or tonic and phasic contractions of the pylorus. In our procedure, not only the blood supply but also the venous drainage of the pyloric region is more sufficient than in PPG. These findings may have contributed to good maintenance of pyloric motility.

The present study demonstrated the safety and effectiveness of our procedures. This procedure could be a new function-preserving gastrectomy that is feasible for treatment of EGC with possible LN involvement. However, further studies including a larger number of patients and longer follow-up periods are essential for a more definitive conclusion.

COMMENTS

Background

Less invasive surgery for early gastric cancer (EGC) has been more commonly employed based on the background of an increase in EGC. Although it has been reported that pylorus-preserving gastrectomy can be performed with D2 dissection, there has been no report on segmental gastrectomy with D2 dissection.

Research frontiers

A sentinel node navigation method has been developed for accurate intraoperative determination of lymph node (LN) metastasis during surgery for EGC.

Innovations and breakthroughs

This is the first report to demonstrate that segmental gastrectomy can be performed with D2 dissection for treatment of EGC.

Applications

The superiority of function-preserving gastrectomy over conventional D2 gastrectomy for EGC in terms of postoperative quality of life is apparent. However, limited LN dissection accompanying function-preserving gastrectomy may reduce the radicality of gastrectomy for EGC. This procedure may be applicable to EGC with possible LN involvement.

Terminology

Segmental gastrectomy is one of the limited gastrectomies in which the central one third portion of the stomach is resected. The sentinel node is generally defined as the lymph node which receives the lymphatic flow first from the tumor.

Peer review

This is an interesting small series and I think it has value. I would like more information concerning the pathological outcomes before accepting the conclusions and suggestions in the discussion.

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BRIEF ARTICLE

Predictive factors for lymph node metastasis in early gastric cancer

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Abstract

AIM: To analyze the predictive factors for lymph node metastasis (LNM) in early gastric cancer (EGC).

METHODS: Data from patients surgically treated for gastric cancers between January 1994 and December 2007 were retrospectively collected. Clinicopathological factors were analyzed to identify predictive factors for LNM.

RESULTS: Of the 2936 patients who underwent gastrectomy and lymph node dissection, 556 were diagnosed with EGC and included in this study. Among these, 4.1% of patients had mucosal tumors (T1a) with LNM while 24.3% of patients had submucosal tumors with LNM. Univariate analysis found that female gender, tumors ⩾ 2 cm, tumor invasion to the submucosa, vascular and lymphatic involvement were significantly associated with a higher rate of LNM. On multivariate analysis, tumor size, lymphatic involvement, and tumor with submucosal invasion were associated with LNM.

CONCLUSION: Tumor with submucosal invasion, size ⩾ 2 cm, and presence of lymphatic involvement are predictive factors for LNM in EGC.

Key words: Early gastric cancer; Lymph node metastasis; Endoscopic treatment; Endoscopic submucosa dissection; Depth of tumor invasion

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INTRODUCTION

The term early gastric cancer (EGC) describes gastric cancers involving the mucosa or submucosa irrespective of the presence of lymph node metastasis (LNM). Radical surgery including lymph node dissection has been the

standard treatment for early gastric cancer; however, LNM has only been associated with approximately 8% to 20% of EGC cases[1-3]. Unnecessary surgery could be avoided and endoscopic treatment might be a consideration in patients with EGC with negligible risk of LNM. Prior studies have demonstrated that the presence of LNM is the most significant factor for survival in patients with EGC[4,5] and usually constitutes the watershed between radical and endoscopic surgery. Identification of LNM cannot be achieved *via* endoscopic ultrasonography or computed tomography because the lymph node size is not a reliable parameter for detection of metastasis[1,2]. Many retrospective studies on EGC have established an indication for endoscopic treatment[6] and the probability of LNM in EGC has been estimated based on macroscopic-endoscopic tumor characteristics and histopathological findings.

According to the treatment guidelines for gastric cancer in Japan[7] the indication for endoscopic treatment such as endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) are patients with non-ulcerated tumors < 2 cm. Several investigators are currently attempting to extend the indication for endoscopic treatment to include: differentiated type, intramucosal cancer without ulcer, > 2 cm in size; differentiated type, intramucosal cancer, ≤ 3 cm in size if ulcerated; and, undifferentiated type, intramucosal tumor without ulcer, ≤ 2 cm in size (Table 1).

While endoscopic treatment for EGC is widely adapted in Japan and Korea using various criteria[4,8-10], it remains uncertain whether these guidelines for EMR/ESD are applicable to patients in areas outside of Japan and Korea. Japan and Korea have the highest gastric cancer rate in the world[11,12] and between 30% and 70% of all gastric cancers are diagnosed as EGCs. In other countries, EGCs account for only about 5% to 15% of all gastric cancers[13,14]. Japan and Korea have national screening programs in which the use of chromoendoscopy could increase the detection rate of EGC. Race, diet, and other factors such as pathologic diagnosis may explain differences in the diagnosis of EGC between Japan and other countries[15,16]; however, comparable data about EGC from other countries is rare or only involve a small case series[17]. The purpose of this study was to identify factors related to LNM in EGC and to elucidate which subgroup of EGC patients could be treated with EMR or ESD instead of radical surgery.

MATERIALS AND METHODS

Between January 1994 and December 2007, 2936 patients underwent gastrectomy for gastric cancer in the Department of Surgery, Chang Gung Memorial Hospital, Taipei, Taiwan, China. Clinicopathological data were obtained from a retrospectively constructed medical database, which had been reviewed by IRB, Chang Gung Memorial Hospital. In total, 556 of these patients were diagnosed with primary EGC and were included in this study. There were 330 men and 226 women with a median age of 62 years (range: 21-89 years).

Table 1 Extended indications for endoscopic mucosal resection/endoscopic submucosal dissection according to the treatment guidelines for gastric cancer in Japan

Differentiated type, mucosal cancer, ulcer (-), > 2 cm
Differentiated type, mucosal cancer, ulcer (+), ≤ 3 cm
Undifferentiated type, mucosal cancer, ulcer (-), ≤ 2 cm

Additional lymph node resection is not necessary when lymphovascular invasion is absent and also when it is not deeper than SM1 (-500 μm).

Poorly differentiated adenocarcinomas, signet ring cell carcinomas, and mucinous adenocarcinomas were classified as undifferentiated tumors. Well and moderately differentiated tubular adenocarcinoma and papillary adenocarcinoma were grouped together as differentiated tumors. Associations between the various clinicopathological factors and the presence of LNM were analyzed to identify risk factors of LNM. These factors included: gender; age (< 65 years or ≥ 65 years), carcinoembryonic antigen (CEA, < 5 ng/mL or ≥ 5 ng/mL), gross appearance, presence of an ulcer, histological type, depth of invasion (mucosa or submucosa), lymphatic involvement, and vascular involvement. Endoscopic macroscopic appearance was evaluated based on the Japanese Classification of Gastric Carcinoma established by the Japanese Research Society for Gastric Cancer[18]. Gross tumor findings were classified into five groups: type I (protruded); Ⅱa (superficially elevated); Ⅱb (flat); Ⅱc (superficially depressed); or Ⅲ (excavated). Macroscopic findings were defined as elevated types (type Ⅰ, Ⅱa, and combined Ⅰ or Ⅱa with Ⅱb), flat type (type Ⅱb), or depressed types (type Ⅱc, Ⅲ, and any combination of Ⅱb, Ⅱc or Ⅲ). When both elevated and depressed types were observed in one lesion, the lesion was defined as mixed type. An ulcer was identified based on the presence of an ulcer or ulcer scar defined endoscopically as converging folds and recognized histologically as a deformity of the muscularis propria or fibrosis in the submucosal layer[19].

Specimens were fixed in 5% formaldehyde and the tumor area and surrounding normal tissue were completely embedded in paraffin. The size (largest diameter) of each carcinoma was measured by the pathologist after fixation. From each block, 2 μm thick sections were cut and stained with hematoxylin and eosin. The depth of infiltration was measured at the deepest point of penetration of the cancer cells[20]. The pT1 category was confirmed as well as the subdivision in pT1a (limited to the mucosa or muscularis mucosa) and pT1b (submucosa).

All dissected lymph nodes were analyzed microscopically for metastatic disease. When necessary, additional lymph node sections were analyzed and special staining was applied. Immunohistochemistry for micrometastasis was not performed.

Statistical analysis

Calculations were performed using SPSS for Windows

Table 2 Clinicopathological features of 556 patients diagnosed with early gastric cancer and univariate analysis of potential risk factors of regional lymph node metastasis

Variables	Positive rate of lymph node metastasis (%)	*P* value
Age (yr)		0.876
< 65	40/288 (13.9)	
⩾ 65	36/268 (13.4)	
Gender		0.012
Male	35/330 (10.6)	
Female	41/226 (18.1)	
Size (cm)		< 0.001
< 2	34/352 (9.7)	
⩾ 2	42/204 (20.6)	
Endoscopic appearance		0.993
Elevated	9/65 (13.8)	
Depressed	43/307 (14.0)	
Flat	16/123 (13.0)	
Mixed	8/61 (13.1)	
Serum CEA (ng/mL)		0.152
< 5	43/336 (12.8)	
⩾ 5	7/32 (21.9)	
Depth of invasion		< 0.001
T1a	12/293 (4.1)	
T1b	64/263 (24.3)	
Histology differentiation		0.759
Differentiated	41/309 (13.3)	
Undifferentiated	35/247 (14.2)	
Vascular invasion		< 0.001
Absence	68/546 (12.5)	
Presence	8/10 (80.0)	
Lymphatic involvement		< 0.001
Absence	44/517 (8.5)	
Presence	32/39 (8.2)	
Ulcer		0.449
Absence	22/182 (12.1)	
Presence	54/374 (14.4)	

(version 11.5K, Chicago, Illinois). The χ^2 test was used to assess potential risk factors of LNM by bivariate comparisons of the categorical variables. Significant factors noted by univariate analysis were subsequently entered into a multivariate logistic regression model for analysis. *P* values < 0.05 were considered to be statistically significant.

RESULTS

EGCs were diagnosed in 18.9% of the gastric cancer cases (556 cases). Of these 556 diagnosed with EGC, 76 (13.7%) had LNM. As shown in Table 2, 293 (52.7%) were intramucosal tumors and 4.1% of these had LNM. In addition, 263 lesions (47.3%) penetrated the submucosa and 24.3% of submucosal tumors had LNM.

Univariate analysis identified that female gender, size ⩾ 2 cm, tumor invasion to the submucosa, presence of lymphatic involvement, and presence of vascular involvement were significantly associated with a higher rate of LNM (Table 2). Tumor size ⩾ 2 cm ($P < 0.008$), deep penetration into the submucosa ($P < 0.001$), and lymphatic involvement ($P < 0.001$) remained significant in multivariate analysis (Table 3).

Table 4 demonstrates the incidence of LNM of our patients fulfilled the criteria used for endoscopic treatment in EGD Japan[7]. In patients without lymphovascular invasion, 42 (tumor size ⩽ 2 cm) and 77 (any tumor size) patients with differentiated intramucosal cancers and no ulceration did not have LMN; intramucosal lesions with ulcer, size ⩽ 3 cm were found in 78 patients, who had no LNM; 3 of 35 patients with undifferentiated intramucosal tumors, no ulceration and size ⩽ 2 cm had LNM.

Table 3 Multivariate analysis of potential risk factors for regional lymph node metastasis

Variables	Odds ratio (95% CI)	*P* value
Gender (female/male)	1.49 (0.35-1.1)	0.163
Tumor size (⩾ 2 cm/< 2 cm)	2.28 (1.20-4.17)	0.008
Vascular invasion (yes/no)	1.86 (0.23-12.65)	0.598
Lymphatic invasion (yes/no)	27.2 (10.3-74.8)	< 0.001
Depth of invasion (T1b/T1a)	4.91 (2.44-9.89)	< 0.001

Table 4 Incidence of lymph node metastasis in our patients fulfilled the criteria used in endoscopic treatment for early gastric cancer in Japan

Criteria	Patient number with lymph node metastasis/total patient	Incidence (%)
Non-ulcerated, differentiated, intramucosal tumor without lymphovascular invasion, ⩽ 2 cm	0/42	0
Non-ulcerated, differentiated, intramucosal tumor without lymphovascular invasion, any size	0/77	0
Ulcerated, intramucosal tumor, without lymphovascular invasion, ⩽ 3 cm	0/78	0
Non-ulcerated, undifferentiated intramucosal tumor without lymphovascular invasion, ⩽ 2 cm	3/35	8.6

DISCUSSION

The incidence of LNM in EGC ranges from 2.6% to 4.8% in mucosal cancers and 16.5% to 23.6% in submucosal cancers[21,22]. In this study the positive rates of LNM in intramucosal and submucosal lesions were 4.1% and 24.3%, respectively, in line with the previous reports[21,22]. Endoscopic treatment for gastric cancer not only preserves gastric function but also helps maintain the patient's quality of life. For patients with EGC, "early" treatment is advocated as the best option for obtaining a complete cure. At present, a correct diagnosis of LNM is impossible during either EMR or ESD. This means that if LNM exists at the time of EMR or ESD, recurrence is very likely. For this reason, multiple patient- and tumor-related variables are currently under investigation as predictors of lymph node involvement[23], particularly in Japan and Korea. Many investigators have suggested possible extended criteria for local treatment[4].

In areas outside of Japan and Korea, the possibility

of using the above-described criteria remains problematic. Japan and Korea have the highest rate of gastric cancer rate in the world[11,12] and that in these countries, between 30% and 70% of all gastric cancers are diagnosed as EGCs. In other areas or countries, EGCs account for only about 5% to 15% of all gastric cancers[13,14,24].

National screening programs and chromoendoscopy could improve the detection rate of EGC; however, race, diet, and other factors such as pathological diagnosis may explain differences in the diagnosis of EGC between Japan and other countries. For example, there are some intestinal-type mucosal cancers in Japan that are not regarded as cancer in Western countries[15,16]. Hölscher *et al*[17] reported that the rate of LNM in mucosal cancer in one European series (6.5%) is higher than reported in Asian countries (2.7%). These differences are even greater if study data are limited to Japan and Korea; however, no difference in submucosal cancer exists (23.9% *vs* 22.1%). Therefore, whether the standard treatment employed in Japan and Korea can be used in other countries remains unknown. Compared with the data generated in this study, the rate of EGC was 18.9%, which is lower than that previously reported in Japan and Korea and similar to the reports from the United States and Europe. Our results revealed that the rate of LNM in intramucosal cancer was 4.1%, higher than the value published from Japan and Korea[17]. This might be explained due to the bias of histology criteria employed in our study (similar to those used in Western countries) and Japan and Korea.

In this study, various clinicopathological factors including gender, age, CEA levels, gross appearances, histological type, invasion depth, lymphatic involvement, and vascular involvement were analyzed for LNM in EGC. Our results showed that female gender was a significant factor in univariate analyses, but was not evident in multivariate analyses. Other research teams reported that being female was associated with LNM in both depressed EGCs and differentiated submucosally invasive EGC[10,25], possibly related to estrogen level[26]. At present, the precise link between gender and LNM remains unclear and further biologic studies are required to explain this effect.

Many studies suggest that serum CEA is an independent risk factor for hematogenous recurrence of gastric carcinoma[27]. Ikeda *et al*[28], for example, reported that stage Ⅱ and Ⅲ gastric cancer patients with higher preoperative CEA levels had frequent liver metastasis. This analysis, however, included all stages of gastric cancer. In the subgroup analysis of EGC in our studies, serum CEA levels were not a significant risk factor for LNM. Whether increased CEA levels have any impact on survival in patients with EGC needs further study.

Some studies have indicated that histological differentiation and ulceration had no significant association with LNM[25,26,29-31]. In contrast, Gotoda *et al*[4] performed a large study including 5265 patients with EGC. They reported that undifferentiated EGC and ulceration were independent factors. Moreover, Ye *et al*[32] assessed 591 patients with undifferentiated EGC and found that poorly differentiated EGCs had higher rates of LNM ($P < 0.001$). In the current study, histological differentiation was not a predictor of LNM, consistent with previous reports[25,26,29-31]. Nonetheless, our results showed that 3 of 35 cases (8.6%) with undifferentiated intramucosal tumors, which were not ulcerative and ⩽ 2 cm in size, had LNM. It is well accepted that in the presence of LNM, only radical gastrectomy provided a chance for curing patients with EGC. Therefore, we suggest that surgical resection with lymphadenectomy instead of endoscopic management should be the treatment choice for undifferentiated EGC.

Many researchers have accepted that depth of tumor invasion is the major factor relating to regional LNM[4,26,30,31] and endoscopic treatment is considered in EGC even if there is minimal submucosal invasion. Nonetheless, some of these researchers still advise against the use of extended criteria even if minimal submucosa invasion is present because rare cases had LNM that met the extended criteria for EMR/ESD[33].

In summary, tumor with submucosal invasion, size ⩾ 2 cm, and presence of lymphatic involvement appear to be significant factors for LNM. Endoscopic treatment might be an alternative in carefully selected EGC patients without predictors for LNM.

COMMENTS

Background

Endoscopic treatment for early gastric cancer (EGC) has been widely adapted in Japan and Korea to avoid unnecessary gastric resection. However, the presence of lymph node metastasis (LNM) which affects patient survival is considered a contraindication of endoscopic management in this situation. This study was designed to analyze the predictive factors for LNM in early gastric cancer.

Research frontiers

Studies have established an indication for endoscopic treatment for EGC and the probability of LNM in EGC is extremely low based on endoscopic and histopathological findings.

Innovations and breakthroughs

Guidelines for endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) remains uncertain in areas outside of Japan and Korea. The authors identify the predictive factors related to LNM in EGC and test the guideline.

Applications

Endoscopic treatment might be an alternative in carefully selected EGC patients without risk factors for LNM.

Terminology

EMR is an endoscopic technique of resection of a lesion that requires the separation of the submucosa using normal saline solution. ESD is a new method of resection, allowing the dissection of the lesion within the thickness of the submucosa or the interface between the submucosa and the muscularis propria.

Peer review

Excellent contribution highlighting the appropriate selection indicators for endoscopic treatment of early gastric cancer indicating that not all early cancers are the same.

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BRIEF ARTICLE

Staging systems for predicting survival of patients with hepatocellular carcinoma after surgery

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Abstract

AIM: To compare the staging systems for stratifying and predicting the prognosis of patients with hepatocellular carcinoma (HCC) after partial hepatectomy (PH).

METHODS: Clinical data about 438 HCC patients who underwent PH from January 1991 to December 2004 at our hospital were retrospectively analyzed. Tumor stage was evaluated following the Chinese tumor node metastasis (TNM) and barcelona clinic liver cancer (BCLC) staging systems, respectively. Survival curves for the HCC patients were plotted using the Kaplan-Meier method and differences were compared by the log-rank test. The accuracy of each system for predicting death of HCC patients was evaluated by calculating the area under the receiver operating characteristic curve.

RESULTS: The HCC patients were classified into stages Ⅰ-Ⅲ, stages Ⅰ-Ⅳ and stages A-C, according to the 3 staging systems, respectively. Log-rank test showed that the cumulative survival rate was significantly different for the HCC patients at 3 Chinese system stages, TNM stages Ⅰ and Ⅱ, TNM stages Ⅲ and Ⅳ, and 3 BCLC stages ($P < 0.05$). However, no significant difference was found in the HCC patients at TNM stages Ⅱ and Ⅲ. The accuracy of the Chinese and BCLC staging systems was higher than that of the TNM staging system for predicting the survival rate of HCC patients.

CONCLUSION: The Chinese and BCLC staging systems are better for stratifying and predicting the prognosis of HCC patients after PH than the TNM staging system.

Key words: Hepatocellular carcinoma; Tumor staging; Prognosis; Survival; Hepatectomy

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INTRODUCTION

Hepatocellular carcinoma (HCC), one of the most common cancers in the world, especially in Eastern Asia, with a poor prognosis[1], is the first leading cause of cancer-related death in the southeast of China[2]. Treatment modalities for HCC are strongly dependent on tumor stage

and the underlying liver diseases. Surgical intervention is the only potentially curative modality for it at present. However, the long-term prognosis of HCC patients remains dismal even after radical excision or liver transplantation[3].

Staging systems are used to define the prognosis of HCC and its treatment[4]. Generally, a better clinical staging system can stratify patients according to their survival time, and is reliable and useful for comparing the curative effects on HCC. Several HCC staging systems have been proposed, such as the Okuda staging system[5], tumor node metastasis (TNM) staging system of the American Joint Committee on Cancer (AJCC)[6], cancer of the liver Italian program (CLIP) scoring system[7], Barcelona clinic liver cancer (BCLC) staging classification[8], Japan integrated staging (JIS) score[9], and Tokyo score[10]. However, no worldwide consensus has been reached on the use of any given HCC staging systems. Therefore, more accurate classification of HCC patients with a homogeneous prognosis would at minimum improve the application of currently available treatment modalities.

The Chinese staging system, established by the Chinese Society of Liver Cancer (CSLC) in 1999[11,12], combines the tumor-related factors and liver function reserve. However, its application in stratifying and predicting the prognosis of HCC patients remains to be defined. This study was to evaluate and compare the Chinese staging system, the AJCC TNM staging system (7th edition) and the BCLC staging system for stratifying and predicting the prognosis of a large cohort of Chinese HCC patients after partial hepatectomy (PH).

MATERIALS AND METHODS

Patients

Four hundred and thirty-eight patients with HCC who underwent PH from January 1991 to December 2004 at our hospital were included in this study. The diagnosis of HCC was pathologically confirmed. Clinicopathological features and survival rates of the patients were analyzed. The study was approved by the Ethical Committee of Sun Yat-Sen Memorial Hospital and in accordance with the Helsinki Declaration of 1975. Written informed consent was obtained from the patients or their guardians.

Tumor stage

HCC was divided into different stages following the criteria for the Chinese, TNM and BCLC staging systems, respectively. The detailed definition and criteria for the Chinese staging system are listed in Table 1.

Treatment

All patients underwent non-anatomical resection. According to the Couinaud's nomenclature for liver segmentation, minor hepatectomy (1-2 segments), major hepatectomy (≥ 3 segments), and wedge resection were performed in 179 (40.9%), 47 (10.9%), and 212 (48.2%) patients, respectively. Of the 438 patients, 24 (5.5%) with unresectable HCC underwent surgery after trans-catheter hepatic arterial chemoembolization (TACE, down-staged). Moreover, HCC patients with inoperable intrahepatic recurrence or extrahepatic metastases received combined therapies, including TACE, radiofrequency ablation (RFA), microwave coagulation therapy (MCT), percutaneous ethanol injection (PEI), and biotherapy or traditional Chinese therapy.

Table 1 Definition and criteria of Chinese staging system for hepatocellular carcinoma (1999)

Stage	Tumor	Thrombi	CLN metastasis	Distant metastasis	Child-Pugh score
Ⅰa	Solitary ≤ 3 cm	No	No	No	A
Ⅰb	Solitary or two ≤ 5 cm in single lobe	No	No	No	A
Ⅱa	Solitary or two ≤ 10 cm in single lobe, or two ≤ 5 cm in bilateral lobes	No	No	No	A
Ⅱb	Solitary or two > 10 cm in single lobe, or two > 5 cm in bilateral lobes	No	No	No	A
	Any	PV-branch, HV or BD	No	No	A
	Any	No	No	No	B
Ⅲa	Any	PV-trunk or IVC	Yes/No	Yes/No	A/B
	Any	Yes/No	Yes	Yes/No	A/B
	Any	Yes/No	Yes/No	Yes	A/B
Ⅲb	Any	Yes/No	Yes/No	Yes/No	C

CLN: Celiac lymph nodes; PV: Portal vein; HV: Hepatic vein; BD: Bile duct; IVC: Inferior vena cava.

Follow-up

During the first 6 mo after operation, the patients were re-examined every 1-2 mo followed by every 3-6 mo. The clinical, laboratory and radiological (abdominal computed tomography scan and chest X-ray) data were collected at each follow-up. Three hundred and ninety-two (89.5%) HCC patients were followed-up until the end of January 2005 or death, while 46 (10.5%) HCC patients were lost during the follow-up. The median follow-up time was 21 mo (range 1-156 mo).

Statistical analysis

Statistical analysis was conducted with the SPSS software package (version 13.0, SPSS, Chicago, IL). Quantitative data were presented as mean ± SE. Survival curves for the HCC patients were plotted using the Kaplan-Meier method and examined by the log-rank test. The accuracy of each system for predicting the 1-, 3-, and 5-year rates of HCC patients was evaluated by calculating the area under the receiver operating characteristic curve. Patients censored before 1, 3 and 5 years were excluded from the analysis. $P < 0.05$ was considered statistically significant.

Table 2 Characteristics of hepatocellular carcinoma patients enrolled in this study

Characteristics	*n* (%)
Sex	
Male	380 (86.8)
Female	58 (13.2)
Age (yr)	
≤ 50	235 (53.6)
> 50	203 (46.4)
Child-Pugh score	
Class A	391 (89.3)
Class B	47 (10.7)
Tumor number	
Single	374 (85.4)
Two	29 (6.6)
Multiple	35 (8)
Tumor size (cm)	
≤ 5	166 (37.9)
> 5	272 (62.1)
Tumor location	
Single lobe	389 (88.8)
Bilateral lobes	49 (11.2)
Capsular invasion	
With	346 (79)
Without	92 (21)
Vascular invasion	
With	84 (19.2)
Without	354 (80.8)
Lymph node metastasis	
With	11 (2.5)
Without	427 (97.5)
Extra-hepatic metastasis	
With	7 (1.6)
Without	431 (98.4)
Histological grade	
G1	129 (29.5)
G2	188 (42.9)
G3	121 (27.6)

RESULTS

Clinicopathological features and survival of HCC patients

The general characteristics of the 438 HCC patients are summarized in Table 2. The mean and median ages of the patients were 50.0 ± 0.6 years and 49.0 years (range: 35-68 years), respectively. By the end of follow up, 223 HCC patients (50.9%) died. The 1-, 3- and 5-year postoperative overall survival rates were 72.2%, 53.5% and 43.3%, respectively, for the patients after PH.

Prognosis stratification according to the three clinical staging systems

The 438 HCC patients were classified into stages Ⅰ-Ⅲ, stages Ⅰ-Ⅳ and stages A-C according to the Chinese, TNM and BCLC staging systems, respectively. The log-rank test showed that the cumulative survival rate was significantly different for the HCC patients at 3 Chinese system stages, TNM stages Ⅰ and Ⅱ, TNM stages Ⅲ and Ⅳ, and 3 BCLC stages (Table 3 and Figure 1, $P < 0.05$). However, no significant difference was found in the HCC patients at TNM stages Ⅱ and Ⅲ ($P > 0.05$).

Table 3 Overall survival rates of hepatocellular carcinoma patients according to their tumor stage

Tumor stage	*n*	Survival rate (%)		
		1-yr	3-yr	5-yr
CS				
Ⅰ	132	0.916	0.760	0.693
Ⅱ	265	0.622	0.426	0.281
Ⅲ	41	0.330	0.126	0.126
TNM				
Ⅰ	271	0.825	0.652	0.548
Ⅱ	67	0.521	0.354	0.216
Ⅲ	82	0.437	0.235	0.153
Ⅳ	18	0.278	/	/
BCLC				
A	178	0.870	0.678	0.602
B	165	0.756	0.574	0.402
C	95	0.375	0.182	0.142

CS: Chinese staging; TNM: Tumor node metastasis; BCLC: Barcelona clinic liver cancer.

Table 4 Accuracy of three staging systems for the death of hepatocellular carcinoma patients 1, 3 and 5 years after partial hepatectomy

System	AUC (95% CI)		
	1-yr death	3-yr death	5-yr death
CS	0.709 (0.658-0.761)	0.703 (0.645-0.761)	0.720 (0.648-0.791)
TNM	0.718 (0.662-0.774)	0.702 (0.646-0.759)	0.695 (0.629-0.760)
BCLC	0.730 (0.675-0.785)	0.701 (0.644-0.757)	0.710 (0.645-0.776)

AUC: Area under the receiver operating characteristic curve; CI: Confidence interval; CS: Chinese staging; TNM: Tumor node metastasis; BCLC: Barcelona clinic liver cancer.

Accuracy of the three clinical staging systems for predicting survival rate of HCC patients

The accuracy of 3 staging systems for predicting the survival rate of HCC patients 1, 3 and 5 years after PH is summarized in Table 4. The accuracy of the Chinese and BCLC staging systems was higher than that of the TNM staging system for predicting the survival rates of HCC patients (Figure 2).

DISCUSSION

HCC is the second most lethal cancer after pancreatic ductal adenocarcinoma, leading to about 600 000 deaths every year worldwide, with nearly 55% of the deaths occurred in China alone[3]. The long term prognosis of HCC patients is extremely dismal and the incidence of HCC is continuously growing globally[1,13,14]. In the southeast of China, HCC is the leading cause of cancer-related death despite aggressive conventional therapies[2]. Surgery remains the most effective treatment of HCC with a curative potential[15,16]. However, HCC patients even after curative resection of the tumor often have a high rate of relapse.

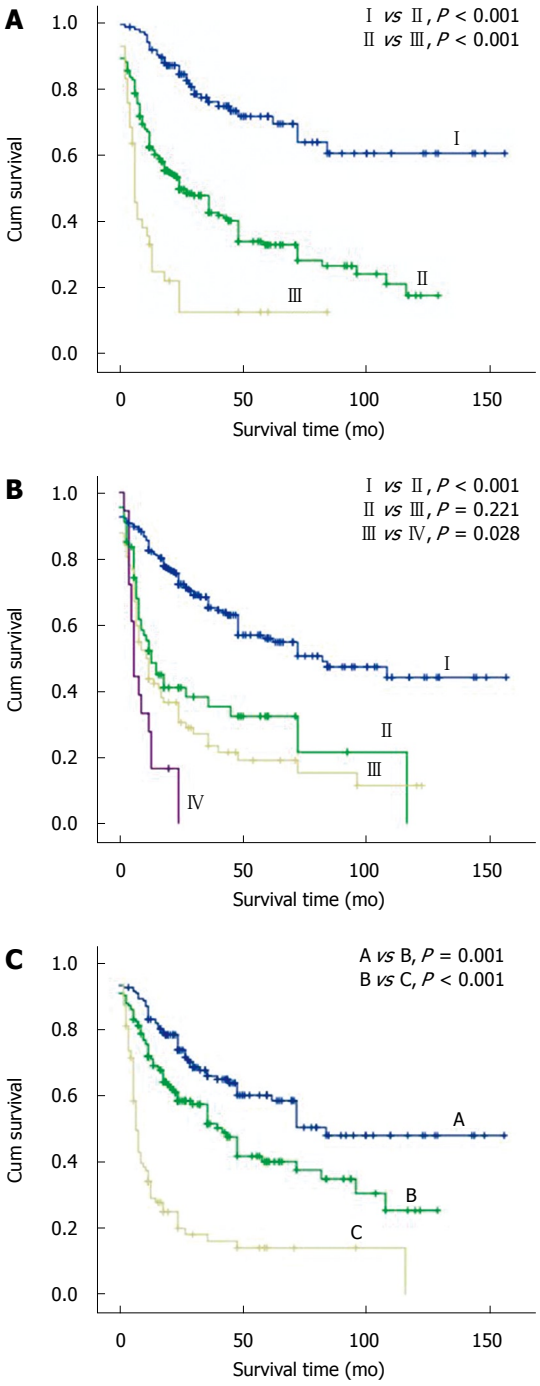

Figure 1 Survival curves for hepatocellular carcinoma patients according to the Chinese staging system (A), tumor node metastasis staging system (B), barcelona clinic liver cancer staging system (C).

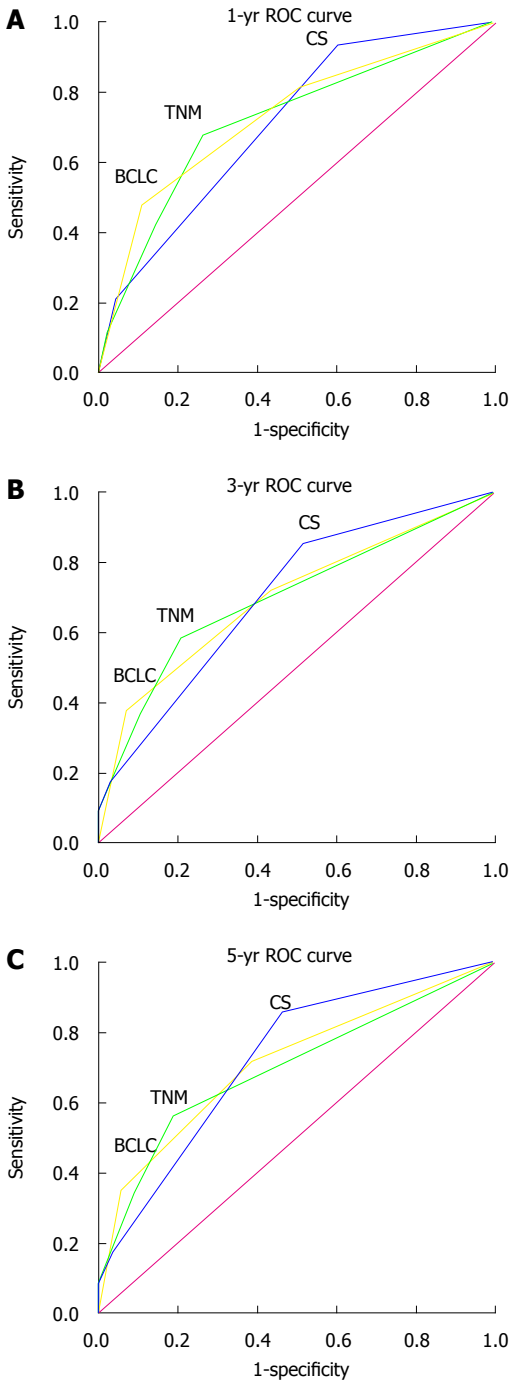

Figure 2 Accuracy of the three staging systems for predicting the 1-year (A), 3-year (B), 5-year (C) survival rates of hepatocellular carcinoma patients. ROC: Receiver operating characteristic curve; CS: Chinese staging; TNM: Tumor node metastasis; BCLC: Barcelona clinic liver cancer.

Therefore, tools that can be used to stratify the prognosis of HCC patients after therapy are urgently needed. Gene-expression analysis has led to the successful molecular classification of HCC according to its prognosis, aetiology and intra-hepatic recurrence[17]. However, it has not been widely accepted due to its high cost.

Staging systems are often used to select primary and adjunctive therapies and to assess their outcome. Generally, a well defined clinical staging system not only can classify HCC patients and predict their survival time, but also can be generally applied globally. Increasing evidence shows that liver function reserve and tumor-related factors can also significantly influence the prognosis of HCC patients[4]. Chen *et al*[18] reported that a better staging system can provide more valid tumor-related factors and liver function parameters. Therefore, both tumor-related features and liver function reserve should be included in the clinical staging systems for HCC[19].

Several clinical staging systems are available for pre-

dicting the prognosis of HCC patients after PH at present. However, no worldwide consensus has been reached on which staging system is the best for predicting the prognosis of HCC patients after surgery[20]. Although the Okuda staging system[5], CLIP scoring system[7], JIS score[9], and Tokyo score[10] are widely used, they have been mostly applied to unresectable HCC, and none is universally accepted[21]. The TNM staging system is the most widely used system worldwide at present[21]. However, the AJCC TNM staging system does not include any measurement of liver functions and is thus not widely used[20]. Llovet *et al*[22] reported that the AJCC TNM staging system fails to adequately stratify HCC patients and predict their prognosis. Lu *et al*[23] also reported that the TNM staging system provides inadequate information for the prognosis of HCC patients.

In the present study, application of the CS system in stratifying and predicting prognosis of a large cohort of Chinese HCC patients (n = 438) after PH was investigated and compared to that of the AJCC TNM and BCLC staging systems. The log-rank test showed that the cumulative survival rate was significantly different for HCC patients at Chinese system stages, TNM stages Ⅰ and Ⅱ, TNM stages Ⅲ and Ⅳ, and 3 BCLC stages, while no significant difference was found in the cumulative survival rate of HCC patients at TNM stages Ⅱ and stage Ⅲ. The accuracy of the Chinese and BCLC staging systems was higher than that of the TNM staging system for predicting the long-term survival (3- and 5-year) rates of HCC patients after PH, indicating that the Chinese and BCLC staging systems are better than the TNM staging system for stratifying and predicting the prognosis of HCC patients after PH. In our study, the AJCC TNM staging system could not accurately stratify the HCC patients with multiple nodules or vascular/peripheral invasion.

In conclusion, the Chinese and BCLC staging systems are better than the AJCC TNM staging system for stratifying and predicting the prognosis of HCC patients after PH. Further studies are needed to establish a single, worldwide staging system that can stratify and predict the prognosis of HCC patients after PH.

COMMENTS

Background

Staging systems are used to define the prognosis and treatment of hepatocellular carcinoma (HCC) patients. Several staging systems are available for predicting the prognosis of HCC patients, but no worldwide consensus has been reached on the use of any given HCC staging systems. Therefore, more accurate classification of HCC patients with homogeneous prognosis is urgently needed.

Research frontiers

Although the Okuda and cancer of the liver Italian program scoring systems, Japan integrated staging score, and Tokyo score are widely used, they have been mostly applied to unresectable HCC patients. The tumor node metastasis (TNM) staging system for HCC does not include any measurement of liver functions and is thus not widely used. The Chinese staging system for stratifying and predicting the prognosis of HCC patients remains to be defined.

Innovations and breakthroughs

The Chinese staging system was studied for stratifying and predicting the prognosis of HCC patients. Furthermore, the applicability of the Chinese staging system was compared with other staging systems for stratifying and predicting the prognosis of HCC patients after partial hepatectomy.

Applications

The results of this study demonstrate that the Chinese staging system can be used for predicting the prognosis of HCC patients after partial hepatectomy.

Peer review

The authors evaluated and compared the Chinese, American Joint Committee on Cancer (AJCC) TNM and Barcelona clinic liver cancer (BCLC) staging systems for stratifying and predicting the prognosis of a large cohort of Chinese HCC patients after partial hepatectomy. The results reveal that the Chinese and BCLC staging systems are better than AJCC TNM staging system for stratifying and predicting the prognosis of HCC patients after partial hepatectomy.

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CASE REPORT

Primary malignant liver mesenchymal tumor: A case report

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Abstract

Primary malignant liver mesenchymal tumor is a rare condition defined as a tumor with vascular, fibrous, adipose, and other mesenchymal tissue differentiation. We report a case of primary malignant liver mesenchymal tumor in a 51-year-old male with anemia, weight loss and hepatomegaly. Finally unconventional liver biopsy and histological manifestation led to the definitive diagnosis.

Key words: Hepatic mesenchymal tumor; Liver biopsy; Hepatic leiomyosarcoma; Hepatic schwannoma

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INTRODUCTION

Primary malignant liver mesenchymal tumor is a very rare tumor, accounting for less than 1% of all hepatic malignancies[1]. Hepatic angiosarcoma, leiomyosarcoma, embryonal sarcoma, schwannoma and lymphoma are the more common mesenchymal tumors. The diagnosis is dependent on histological imaging. However, some cases of multiple nodular lesions can only be diagnosed by percutaneous liver biopsy (PLB) without surgery. We present a case of malignant liver mesenchymal tumor in an adult and its diagnosis and treatment were discussed.

CASE REPORT

A 51-year-old Chinese male was referred to our hospital with a 2-mo history of mild abdominal pain, an abdominal mass in the upper quadrant, fatigue and progressive weight loss. His symptoms gradually worsened with fatigue 1 wk after onset of the disease. He had no fever, cough or skin lesions in the past 2 mo and no family history of liver and autoimmune diseases. Physical examination revealed mild pale conjunctiva and skin, and enlargement of the liver without percussion pain.

His complete blood hemoglobin was 58 g/L. Stool occult blood test was negative. Liver functional test showed that his alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ glutamyl tranzpehtidaze, total bilirubin, direct bilirubin, and albumin were 81 U/L, 86 U/L, 224 U/L, 73 U/L, 23.55 μmol/L, 11.74 μmol/L, 30.5 g/L, respectively. Series hepatitis markers (hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, Epstein-Barr virus, and cucumber mosaic virus) were negative. α-fetoprotein, carcinoembryonic antigen and CA19-9 concentrations were also normal. Human immunodeficiency virus and syphilis antibodies were negative. Coagulation profile was normal.

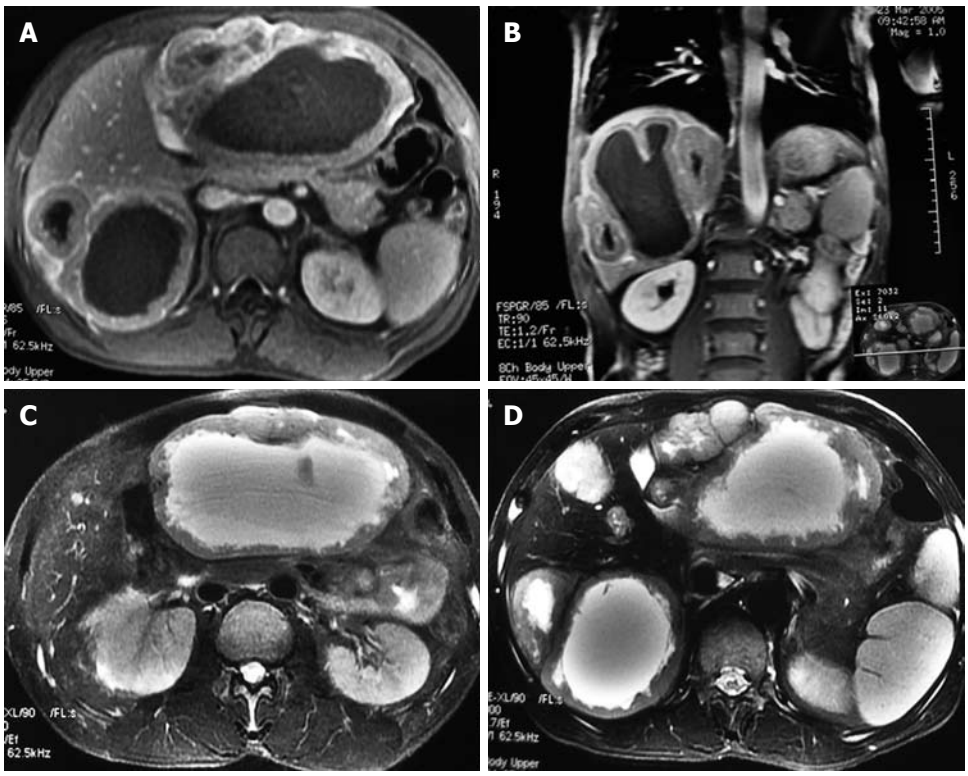

Figure 1 Axial fat-suppressed T2-weighted turbo spin echo magnetic resonance imaging showing two large lesions (A) and several small cyst-like lesions (B) in liver, and coronal and transverse T1-weighted imaging showing thickened wall of lesions with a ragged appearance (C) and enhancement (D) in liver.

Ultrasound and magnetic resonance imaging (MRI) were used as the initial diagnostic tools. Abdominal ultrasound revealed a solid mass in the liver with a low echo, a sharp border, a rich blood supply and liquefaction necrosis. The maximum diameter of the mass was 13.9 cm. Abdominal MRI showed multiple diffuse abnormal signals suggestive of a possible malignant tumor. Axial fat-suppressed T2-weighted turbo spin echo imaging showed two larger and several smaller well-margined cyst-like lesions in the liver (Figure 1A and B). Coronal and transverse T1-weighted imaging demonstrated thickened wall of lesions with a ragged appearance and enhancement (Figure 1C and D). Endoscopy showed duodenal ulcer (S2 stage), normal colon and rectum.

PLB was performed under ultrasonography (US) guidance using a Bard biopsy gun (18-gauge cut needle) to make a final diagnosis of the lesions in liver. During biopsy, bloody liquid was observed in the lesion. Microscopic examination confirmed that the bloody liquid contained a large number of erythrocytes. However, pathology of the liver only showed a spindle cell tumor. The possible reasons are as follows. First, the liver specimen was too small because the lesion contained only fluid and was surrounded by a very thin wall. Second, the location of tumor and its hardness limited the adjustable puncture angle.

To confirm the diagnosis of this patient, another unconventional liver biopsy was performed with gastroscopic biopsy forceps as follows.

At beginning of the procedure, a local anesthetic agent (5% lidocaine) was injected subcutaneously through a 25-gauge needle. Next, a 15 cm long 18 gauge puncture needle was passed into the cyst under US guidance. After the local skin and subcutaneous tissue were dilated, a PTC exchange stainless steel guide wire with a flexible tip was introduced into the cyst cavity through the needle, and then the needle was withdrawn. A 5-French catheter was plated into the cyst using the Seldinger technique. Finally, gastroscopic biopsy forceps was passed into the cyst through the catheter to get the liver tissue sample. The histological results indicated that spindle-shaped tumor cells were well-oriented, arranged in bundles and clustered in some part of the regional tumor. Nuclei with rare mitosis were elongated in a rod-like shape with different sizes and blunt ends. The final diagnosis was established as a low grade malignant liver mesenchymal tumor (Figure 2).

The patient was suggested to receive hepatic arterial chemoembolization. Selective digital subtraction angiography in the early and later phases showed lesions surrounded by abnormal tortuous tumor arteries (Figure 3A) and patchy enhancement (Figure 3B), respectively, in different areas. Then, 5-Fu (750 mg), mitomycin (5 mg), pyrazine imidacloprid Star (30 mg), and super-liquid iodized oil (5 mL) were infused into the tumor arteries. After treatment, liver function of the patient was improved and his hemoglobin level increased (Table 1). The patient died 2 years after treatment.

DISCUSSION

In this paper, we presented a case of multiple nodular cys-

Table 1 Changes in serum biochemical index before and after treatment

	WBC ($\times 10^9$/L)	HGB (g/L)	PLT ($\times 10^{12}$/L)	ALT (U/L)	AST (U/L)	TBIL (μmol/L)	ALB (g/L)
Diagnosis	10.44	58	294	81	86	23.55	30.5
Treatment	6.24	86	241	17	24	20.74	34.1

WBC: White blood cell count; HGB: Hemoglobin; PLT: Platelet count; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; ALB: Albumin.

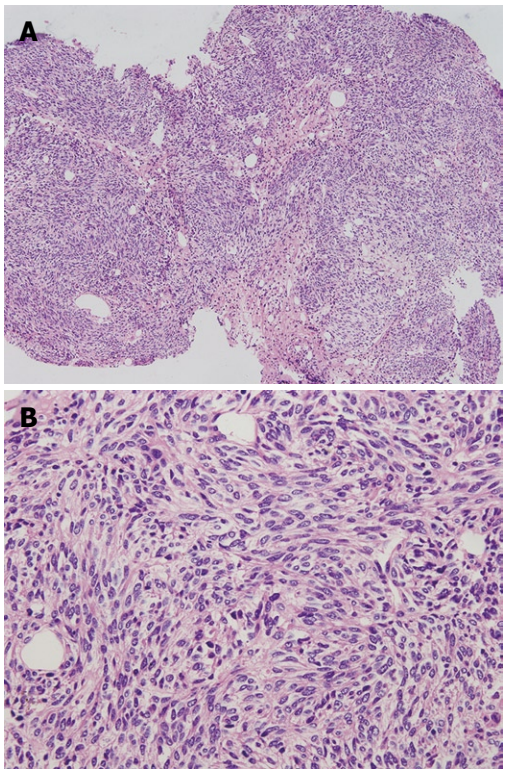

Figure 2 Hematoxylin and eosin staining showing a low grade malignant liver mesenchymal tumor under the magnification × 10 (A) and × 40 (B).

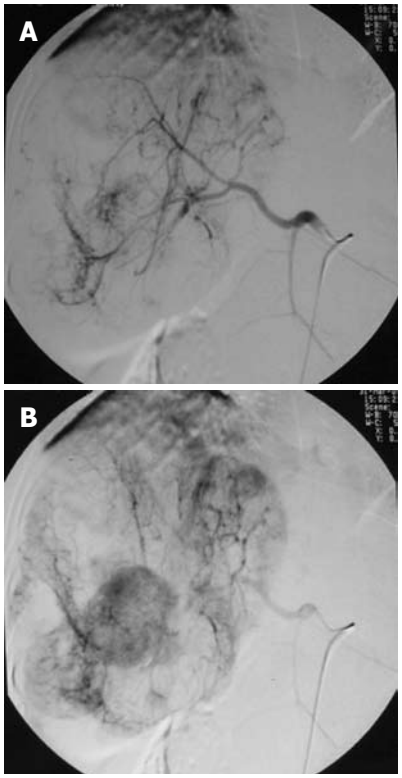

Figure 3 Selective digital subtraction angiography showing abnormal tortuous arteries in the early phase (A) and patchy enhancement in later phase (B) in different areas.

tic lesions in liver. The final diagnosis was established as a low grade malignant liver mesenchymal tumor. However, HE staining could not show the source of mesenchymal cells. Further immunohistochemistry staining with Vimentin, Desmin and α-SMA was performed, which could not sill show the source of mesenchymal cells.

Primary malignant liver leiomyosarcoma and schwannoma are both rare tumors in the liver. Primary liver leiomyosarcoma is a rare malignancy involving the liver, occurring as a primary liver sarcoma in patients without any underlying disorder, and its incidence increases as a primary tumor in immunodeficiency patients[2]. Its usual clinical presentation is painful hepatomegaly or epigastric mass[3]. Malignant liver schwannoma is the most common soft tissue sarcoma in adults, but primary liver schwannoma is extremely rare. Only 12, 8, and 1 cases of benign, malignant, and semimalignant liver schwannoma are available from the literature worldwide[4-12]. Hepatic schwannoma is usually associated with neurofibromatosis. However, two cases of malignant liver schwannoma without neurofibromatosis have been reported[9,11].

Unfortunately, it is difficult to make the diagnosis of malignant liver mesenchymal tumor because both its clinical presentation and imagine are nonspecific.

The common symptoms and signs of patients with malignant liver mesenchymal tumor include abdominal pain, weight loss, weakness, loss of appetite, vomiting, enlargement of the liver, ascites, and jaundice, which lack of specificity in differential diagnosis between benign and malignant mesenchymal tumors.

Furthermore, imaging studies, such as MRI scan, computed tomography (CT) scan and angio photography are the commonly used methods to identify the characteristics of liver tumor. However, imaging findings of liver mesenchymal tumors, including leiomyosarcoma and schwannoma, are nonspecific and infrequently reported[13-19]. It is quite difficult for MRI and CT scan to differentiate primary liver mesenchymal neoplasms from other liver malignancies, although they should be included in differential diagnosis when MRI or CT scan demonstrates hepatic lesions without characteristics of hepatocellular carcinoma,

especially in patients with no extrahepatic primary malignancies.

The diagnosis of malignant liver mesenchymal tumor depends on histological change in either needle or open biopsy, while metastatic status is facilitated by the presence of extrahepatic primary tumor.

Liver biopsy is an important diagnostic tool and helps make therapeutic decision for liver tumor. Open biopsy is a major surgical procedure for liver tumor. PLB under ultrasound or CT guidance is a safe and almost painless procedure for lesions with a soft tissue component or located close to vital structures[20]. Most reported cases of cystic liver mesenchymal tumor were diagnosed by open liver biopsy. In this case, uncommon PLB method was used instead of the routine PLB to make the final diagnosis, avoiding injury and complications of open biopsy. Minimally invasive treatment devices, such as gastroscopic biopsy forceps and catheters used in liver cancer intervention, make the new PLB method possible, which is safe and reliable and can thus be used in diagnosis of cystic liver lesions.

In conclusion, mesenchymal liver tumor is rare in adults and cross-sectional findings are varied, which can be diagnosed with the uncommon PLB method.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
San Francisco, CA, United States
The Medical Management of HIV/AIDS

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Instructions to authors

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There are unstructured abstracts (no more than 256 words) and structured abstracts (no more than 480). The specific requirements for structured abstracts are as follows:

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Key words

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Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use

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Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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Format

Journals

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

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

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 Pixudiarrhoea. *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).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H. pylori*, *E coli*, *etc.*

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Contents

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Contents

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EDITORIAL

Sphincteroplasty for fecal incontinence in the era of sacral nerve modulation

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Abstract

The role of sphincteroplasty in the treatment of patients with fecal incontinence due to anal sphincter defects has been questioned because the success rate declines in the long-term. A new emerging treatment for fecal incontinence, sacral nerve stimulation, has been shown to be effective in these patients. However, the success rate of sphincteroplasty may depend of several patient-related and surgical-related factors and the outcome from sphincteroplasty has been evaluated differently (with qualitative data) from that after sacral nerve stimulation (quantitative data using scoring systems and quality of life). Furthermore, the data available so far on the long-term success rate after sacral nerve modulation do not differ substantially from those after sphincteroplasty. The actual data do not support the replacement of sphincteroplasty with sacral nerve stimulation in patients with fecal incontinence secondary to sphincter defects.

Key words: Fecal incontinence; Sacral nerve stimulation; Sphincteroplasty

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INTRODUCTION

Sphincteroplasty was first described by Lockhart-Mummary[1] in 1923, who reported only on the end-to-end apposition of the margins of the damaged anal sphincter, however, the operation become popular following a publication by Parks *et al*[2], who first described the overlapping sphincteroplasty. Since then, this operation is generally believed to be the treatment of choice for incontinent patients with external anal sphincter defects in whom conservative management has failed. However, this operation has recently been questioned because of its long-term success rate, which like many other operations for functional diseases of the gastrointestinal tract, declines with time. On the other hand, the new procedure of sacral nerve modulation has been demonstrated to benefit patients, even those with damaged anal sphincter, and has raised the question of whether sphincteroplasty still has a role in the management of patients with fecal incontinence after sphincter damage. In this editorial, the reason for the failure of sphincteroplasty and the matter of using sacral nerve stimulation (SNS) instead of sphincteroplasty in patients with sphincter damage are discussed.

PROGNOSTIC FACTORS OF SPHINCTEROPLASTY

Sphincteroplasty is usually indicated after obstetric damage to a normal anal sphincter during childbirth or after an iatrogenic lesion to cure an abscess or a complex anal fistula. It is uncertain whether the etiology of the sphincter lesion can affect the outcome. One of the few papers dealing with this topic suggests that patients with surgical trauma do better than those with obstetric trauma[3]. However, we did not find similar results in the Italian registry of fecal incontinence (data not shown). Of course, the extent of sphincter damage plays an important role in the outcome. It is easier and probably more effective to close a small gap of 30° than a gap of 120°, and in most of the papers dealing with this operation, patients with a gap in the anal defect greater than 120° are usually excluded from this procedure[4]. Another possible factor influencing the success rate after sphincteroplasty could be the occurrence of pudendal neuropathy. Nowadays, the pudendal nerve terminal motor latency test is not generally considered to be predictive of the outcome of several procedures for fecal incontinence such as sacral nerve modulation, however, most of the papers on this topic[5-9] demonstrated that patients without pudendal neuropathy did significantly better than patients with neuropathy (Table 1). The problem is that, almost all these studies are retrospective and it is not possible to demonstrate whether neuropathy existed prior to surgery. Another important factor potentially affecting the outcome is the age of these patients. The literature shows that patients less than 40 or even 50 years old do much better than older patients[10].

Further possible factors which can influence the outcome of this operation are related to the surgical technique. There are many tips on the surgical techniques used which could play a role in the outcome of these patients. For example, early *vs* delayed repair. A recent study[11] demonstrated that early repair, whenever possible, has better cost-effectiveness than delayed repair. In addition, a study by Sultan *et al*[12] indicated that suturing the internal and external anal sphincter separately could give better results, although this data has never been confirmed.

It is generally believed that sphincter overlap is the preferred way to perform a sphincteroplasty. However, in the literature there are only 2 papers on this topic. In particular, the study by Fitzpatrick *et al*[13], which had a very short follow-up, found no difference in outcome. However, the study by Fernando *et al*[14], in a large group of patients, found a significantly better outcome at one year follow-up when the overlapping technique was used.

Other surgical details include the use of resorbable or unresorbable sutures. Some older studies on sphincteroplasty were performed using catgut to approximate the sphincter margins, and this is of major importance when we look at the long-term results. The literature suggests that the use of PDS or prolene is better than Vicryl or Dexon because they need a longer time to be resorbed[15].

Some authors[16] have advocated the need for a diverting stoma to protect the sphincteroplasty, but there is no evidence for this; on the contrary, there is evidence of morbidity following stoma closure and stoma-related disability[17]. Almost all colorectal surgeons do not use a diverting stoma or bowel confinement after a sphincteroplasty and usually these patients can resume oral feeding immediately after surgery[18].

Another interesting factor which potentially affects the outcome of sphincteroplasty, is the puborectalis sling contraction. Roche's group in Ginevra[19] demonstrated that patients with good function of the puborectalis sling measured by perianal echography do better. This means that patients who still have some functionality of pelvic floor muscles have a better chance of obtaining a good result after sphincteroplasty.

Table 1 Effects of pudendal neuropathy on the outcome of sphincteroplasty for fecal incontinence

Author	*n*	Success without neuropathy (%)	Success with neuropathy (%)	*P* value
Laurberg *et al*[5], 1988	19	42	5	< 0.05
Londono-Schimmer *et al*[6], 1994	94	60	14	< 0.001
Sitzler *et al*[7], 1996	29	48	24	< 0.05
Chen *et al*[8], 1998	12	75	50	< 0.05
Gilliland *et al*[9], 1998	77	62	17	< 0.01

Table 2 Short term outcome after sphincteroplasty for fecal incontinence

Author	*n*	Obstetric/ surgery	Results (%) excellent/good	Fair	Poor
Fleshman *et al*[20], 1991	55	100	72	22	6
Wexner *et al*[21], 1991	16	100	76	19	5
Engel *et al*[22], 1994	55	100	79	-	21
Oliveira *et al*[23], 1996	55	99	71	9	20
Felt-Bersma *et al*[24], 1996	18	94	72	-	28
Nikiteas *et al*[3], 1996	42	88	60	17	24
Sitzler *et al*[7], 1996	31	87	74	-	26
Ternent *et al*[25], 1997	16	100	44	31	25
Zorcolo *et al*[26], 2005	93	100	65	9	27
Barisic *et al*[27], 2006	65	86	74	17	9

FUNCTIONAL OUTCOME AFTER SPHINTEROPLASTY

Patients with fecal incontinence have a normal life-expectancy, so we must look at the long-term results rather than the early results. Table 2 lists the studies dealing with the short-term results of sphincteroplasty, in most cases after obstetric trauma, indicating a very interesting rate of excellent/good results for a pooled good outcome of about 69%. However, if we look at the long-term results, after a follow-up ranging from 5 and 10 years (Table 3), the number of excellent/good results falls to 46%.

Why does the success rate deteriorate with time after sphincteroplasty? Some suture breaks in the muscle can occur in the postoperative period, and other patients may have an undetected cause of fecal incontinence. Furthermore, it is supposed that in women, a deteriora-

Table 3 Long term outcome after sphincteroplasty for fecal incontinence

Author	*n*	Median FU (mo)	Results (%) excellent/good	Fair	Poor
Pezim *et al*[28], 1987	40	67	62	-	38
Londono-Schimmer *et al*[6], 1994	94	59	50	26	24
Malouf *et al*[29], 2000	46	77	50		
Halverson *et al*[30], 2002	49	69	14	32	54
Vaizey *et al*[31], 2004	21		52	10	38
Bravo Gutierrez *et al*[32], 2004	130	120	22	19	57
Zorcolo *et al*[26], 2005	62	70	45	10	45
Barisic *et al*[27], 2006	65	80	48	13	39
Maslekar *et al*[33], 2007	64	84	80		20
Oom *et al*[34], 2009	120	111	38	23	40

tion in muscle trophism and innervation occur with age, particularly after the menopause due to the fall in estrogen levels[35].

WHAT TO DO IN CASE OF EARLY OR LATE FAILURE OF SPHINCTEROPLASTY?

The first question is whether the same operation can be re-done. In the literature, there are two papers on this topic[31,36], both claiming that 50% of these patients can recover their continence after a repeat sphincteroplasty, and that continence can be maintained in the long-term.

SACRAL NERVE STIMULATION *VS* SPHINCTEROPLASTY

In 1995, Matzel *et al*[37] opened a new chapter in coloproctology, showing that sacral nerve stimulation can benefit patients with fecal incontinence. Since then, the indication for this procedure has been extended, even to patients with sphincter defects which were originally excluded. In the literature, there are already 7 papers[38-44] published on this topic showing that there is a very interesting percentage of early success with SNS without doing anything to the damaged sphincter. The question now is, are we justified in skipping sphincteroplasty in the ideal "algorithm" to treat patients with sphincter defects by performing SNS directly? There are several issues that make this option questionable because the data available on sphincteroplasty and SNS have been poorly compared. There are two major issues that must be considered; the short- *vs* the long-term outcome and how the success rate is measured. Four papers dealing with the first topic were published in 2009 (Table 4)[45-48].

When the data was amalgamated a 58% success rate in the long-term was observed. This was quite unexpected after the enthusiasm surrounding the early results of SNS. However, is this long-term outcome data truly comparable with those of sphincteroplasty? When dealing with SNS, we refer to mixed causes of incontinence, in both sexes, and, most importantly, the criterion used to define success is more than a 50% reduction in major incontinence episodes. When we deal with sphincteroplasty, we look at patients with only one defect causing incontinence, the sphincter defect. Almost all patients are female and the criteria used to define success have been categorical and qualitative such as incontinence to solids, liquids or flatus. A good way of measuring the outcome of surgery for fecal incontinence would be patient satisfaction and quality of life, but few papers focus on these measurements. Most papers on sphincteroplasty just classify the outcome into categories of incontinence. In contrast, in all of the papers on SNS, incontinence scores are used. However, even in these cases confusion can arise due to the use of different incontinence scores (Wexner Score[49], Vaizey's score[50], Pescatori's score[51], and many others), making the results between SNS and sphincteroplasty difficult to compare. Furthermore, the criteria for defining success used in SNS studies (< 50% of incontinence episodes) have never been adopted in sphincteroplasty studies.

Table 4 Long term outcome after sacral nerve stimulation for fecal incontinence

Author	*n*	Success (%)	Length of follow-up (yr)
El-Gazzaz *et al*[45], 2009	22	42	2.5
Altomare *et al*[46], 2009	52	62	7
Matzel *et al*[47], 2009	12	75	9
Vallet *et al*[48], 2010	32	53	3

Another argument based on the definition of success is related to the treatment of fecal incontinence, as the success of the techniques is defined by the statistically significant reduction of incontinence scores, however, this information does not give us what we need to know: how many of our patients have had their problems fixed, not the statistical changes in the global scores.

A very interesting recent paper[34] reviewed 160 patients who had undergone sphincteroplasty, with a very long follow-up of about 10 years. The outcome was classified using the two main systems: categorical (excellent, moderate and poor results) and quantitative (number of incontinence episodes/week). The authors found that 37% of patients had an excellent/good result, 23% had a moderate result and 40% had a poor result. However, the group of patients with a moderate result had more than a 50% reduction in incontinence episodes. This means that, if we analyze the data on sphincteroplasty using the criteria generally adopted for patients undergoing SNS, the long-term success rate is 60%, which is at least comparable to that of SNS.

CONCLUSION

From the literature, overlapping sphincteroplasty can achieve satisfactory long-term results, and, at the moment, they are at least comparable to SNS. Repeat sphincteroplasty may be performed and should be considered as the treatment of choice in the case of failure of previous sphincter repair. Sacral Nerve Stimulation is an effective,

fascinating but expensive technique which should be offered to patients who have failed a previous surgical attempt to repair the sphincter.

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EDITORIAL

Non-invasive markers of gut wall integrity in health and disease

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Abstract

The intestinal mucosa is responsible for the absorption of nutrients from the lumen and for the separation of the potentially toxic luminal content (external environment) from the host (internal environment). Disruption of this delicate balance at the mucosal interface is the basis for numerous (intestinal) diseases. Experimental animal studies have shown that gut wall integrity loss is involved in the development of various inflammatory syndromes, including post-operative or post-traumatic systemic inflammatory response syndrome, sepsis, and multiple organ failure. Assessment of gut wall integrity in clinical practice is still a challenge, as it is difficult to evaluate the condition of the gut non-invasively with currently available diagnostic tools. Moreover, non-invasive, rapid diagnostic means to assess intestinal condition are needed to evaluate the effects of treatment of intestinal disorders. This review provides a survey of non-invasive tests and newly identified markers that can be used to assess gut wall integrity.

Key words: Intestinal damage; Markers; Human; Gut wall integrity

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COMPOSITION OF THE GUT WALL

The gut wall forms a physical/anatomical and immunological barrier. The physical/anatomical barrier of the gut is formed by a monolayer of epithelial cells, originating from multipotent stem cells present in the crypt. The epithelial cells, together with the lamina propria, form the mucosa of the intestine[1,2]. The epithelial stem cells differentiate into four major epithelial cells: (1) the absorptive enterocytes, which make up > 80% of all small intestinal epithelial cells; (2) the goblet cells, which produce a variety of mucins and trefoil peptides; (3) the enteroendocrine cells, which export peptide hormones; and (4) the Paneth cells, which secrete a wide variety of antimicrobial peptides.

Tight junctions (TJ) are the major complexes responsible for the adherence of intestinal epithelial cells to one another and in this context are an important part of the intestinal barrier[3] (Figure 1). Multiple proteins build up TJ: occludins and members of the claudin family are the major sealing proteins. The sealing proteins interact with cytoplasmic proteins, including zonula-occludin proteins, functioning as adaptors between the TJ pro-

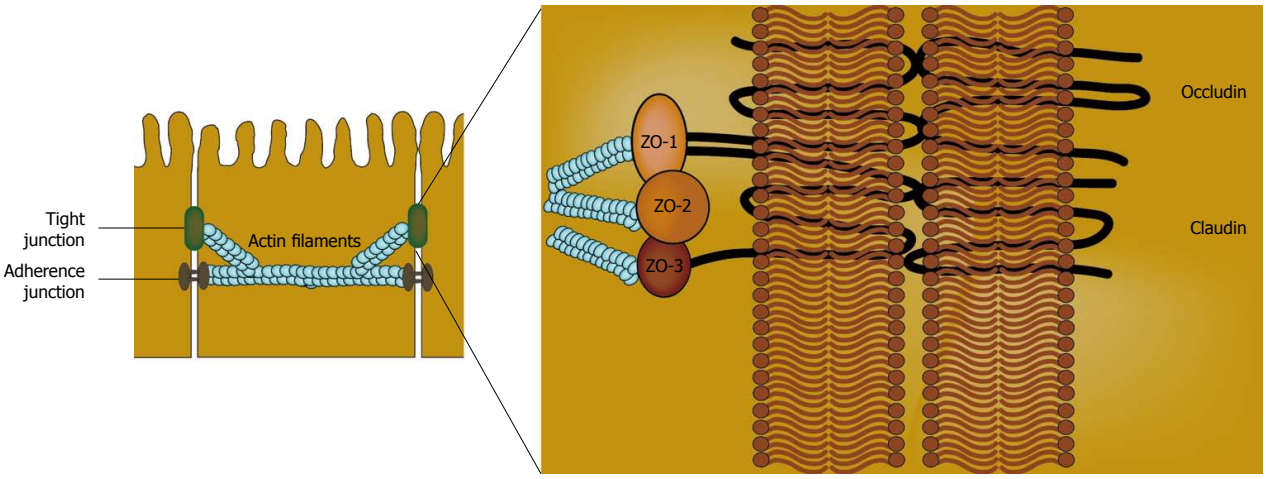

Figure 1 Composition of tight-junctions [in green (left) and in detail (right)] between neighbouring enterocytes (in yellow).

teins and actin and myosin contractile elements within the cells[3]. Breakdown of this barrier potentially leads to translocation of luminal antigens, microbiota, and their toxic products into the circulation. The layers next to the mucosa, the submucosa, muscularis and serosa, are not in direct contact with the lumen. Injury of these layers can result in passage of the luminal content into the abdominal cavity, which is a serious complication.

Next to the physical/anatomical barrier, there is also an immunological barrier. The intestinal epithelium is not merely a static barrier, but participates in immunosurveillance[1,2]. Enterocytes are considered to actively participate as innate immune sensors of microbial pathogens and commensal organisms[4]. Host recognition of microbial components is achieved by so-called pattern recognition receptors (PRRs), such as the NOD-like and Toll-like receptors. Muramyl dipeptide, derived from peptidoglycan, is present in the cell wall of virtually all bacteria and is recognized by NOD2, a PRR expressed in intestinal epithelial cells, including Paneth cells[4]. Crypt Paneth cells secrete defensins, which are antimicrobial peptides, into the villous crypt, maintaining its sterility. Moreover, we recently reported that Paneth cells are equipped with the molecules that recognize and signal endotoxin, one of the most potent immunostimulatory products derived from Gram-negative bacteria[5]. Continuous antimicrobial protection of the crypt is of crucial importance, as the pluripotent stem cells are located there. Damage to stem cells has severe consequences for the maintenance of the homeostasis of normal gut epithelium[6]. Goblet cells secrete mucus, a composition of glycoproteins and water, which provides a filter overlying the intestinal epithelium. Additionally, goblet cells secrete trefoil peptides, small proteins needed for epithelial growth and repair. Furthermore, gut-associated lymphoid tissue is present in the lamina propria and provides immune surveillance. Sampling of luminal antigens occurs by M-cells and dendritic cells, which present antigens to T and B cells, thereby inducing an effector immune response. This response includes secretion of large amounts of IgA by plasma cells. This secretory IgA covers the mucosal surface and has a major role in excluding antigens from passing the epithelium[1,2].

MARKERS OF GUT WALL INTEGRITY

Intestinal permeability

Intestinal permeability is frequently assessed using oral ingestion of small to large-sized probe molecules and measurement of their urinary excretion[7,8]. Large molecules are thought to traverse the epithelium by paracellular pathways *via* tight junctions between the enterocytes. Permeability of the tight junctions presumably increases in diseased or damaged mucosa, resulting in increased absorption of large molecules. Small molecules are postulated to pass predominantly by transcellular pathways through aqueous pores in the enterocyte membranes that are too small to permit the passage of large molecules. The ratio of urinary excretion of the relatively large molecule is compared with that of the relatively small molecule. When a large and small molecule are combined in the test solution at a fixed concentration ratio, the effects of variables, such as gastric emptying, intestinal transit time, and renal clearance will apply equally to both. Thus, the urinary excretion ratio of these two molecules is expected to be influenced only by the difference in gut permeability for each molecule.

Disaccharides (lactulose) or Poly-ethylene-glycol (PEG)-3350 are frequently used as orally ingested large molecules, while monosaccharides (mannitol, L-rhamnose) or PEG-400 are used as small molecular probes[8]. Subsequently, the renal excretion of the two probes is monitored over a defined interval (mostly 5 h), and permeability is then expressed as the quotient (ratio) of the urinary recovery of the large molecule divided by the small molecule[8]. It is assumed that the probes used are non-fermentable by bacteria in the gastrointestinal lumen and that they are not metabolized in the body. These molecules are also supposed to be excreted in urine in proportion to the amount that has been absorbed through the intestinal mucosa[8]. Thus far, contrasting results have been reported for intestinal permeability tests using dual probe molecules in several studies[7,9]. This is mainly attributed to a number of assumptions that have to be made to interpret the test-results[8,9]. In particular, pathways of intestinal permeation of the different molecules

and the mechanisms by which permeability is altered are as yet incompletely understood.

Translocation of bacteria and their products

Breakdown of the mucosal barrier potentially leads to translocation of microbiota or their toxic products. Two promising plasma markers, reflecting translocation of bacteria or their products, are D-lactate and endotoxin lipopolysaccharide (LPS), which are metabolic products or components of the commensal bacteria of the gastrointestinal tract. D-lactate is only produced by bacteria as a product of bacterial fermentation[10]. Baseline levels of D-lactate in healthy subjects are very low. Increased levels of D-lactate have been correlated with conditions in which the number of bacteria elevates rapidly, including in patients with bacterial overgrowth due to infection, short bowel syndrome, and mesenteric ischaemia[11]. LPS, the major constituent of the outer membrane of Gram-negative bacteria, is released by Gram-negative bacteria when replicating or dying. Increased circulating LPS levels have been related to an impaired mucosal barrier. The presence of LPS can be measured directly in blood, e.g. by the Limulus Amoebocyte Lysate assay[12]. In addition, anti-LPS antibodies can be measured by endotoxin-core antibody (EndoCAb), an indirect measurement of LPS leakage into the circulation[13]. A drop in levels of circulating anti-LPS antibodies is considered to indicate consumption of antibodies to LPS by exposure to LPS[14].

Transmural damage

Any part of the gastrointestinal tract may undergo damage to all layers of the GI wall from a variety of causes, releasing gastric or intestinal contents into the peritoneal cavity, which can cause peritonitis. Symptoms develop suddenly, with severe pain followed shortly by signs of (septic) shock. If a perforation is noted, immediate surgery is necessary, because mortality from peritonitis increases rapidly. The diagnosis of transmural damage (i.e. perforation) of a gastrointestinal organ usually depends on the detection of free intraperitoneal air, which is most often located in the right subphrenic space. Traditionally, a chest X-ray and a plain abdominal X-ray in the upright position, or, more recently, ultrasonography, are the diagnostic tools used to detect free air. However, the sensitivity of these tools is < 80%. Currently, a computed tomography scan is sometimes performed, which can detect free intraperitoneal air as well as small fluid collections and subtle tissue-infiltration at different locations.

Splanchnic perfusion

Numerous clinical conditions are accompanied by a reduced splanchnic blood flow, including vascular disease, major surgery, and various types of shock. Prolonged hypoperfusion of the splanchnic region will inevitably lead to hypoxic tissue injury. Furthermore, the splanchnic region is an important source and target of inflammatory mediators, which have a major impact on both systemic and regional blood flow and tissue function[15]. Gut mucosal perfusion can be invasively measured by gastric tonometry. Gastric tonometry assesses the pCO_2 in the gastric mucosa, taking into account that an increase in tissue CO_2 production accompanies anaerobic metabolism, signifying the effectiveness of regional splanchnic perfusion[16].

Functional enterocyte mass

Functional enterocyte mass is reflected by levels of circulating citrulline, an amino acid not incorporated into proteins[17]. Differentiated small intestinal enterocytes specifically produce citrulline from glutamine, and are responsible for the major part of the total amount of circulating citrulline[18]. Loss of small bowel epithelial cell mass results in declined circulating levels of citrulline, as is shown in haemopoietic stem cell transplant recipients suffering from severe oral and gastrointestinal mucositis following intensive myeloablative therapy[17].

Tight junctions

Currently, invasive intestinal biopsies provide the only possibility to detect tight junction breakdown. Endoscopy for biopsy is a time-consuming and invasive procedure.

In a recent translational study, using both a rat hemorrhagic shock model and a human setting of patients with active inflammatory bowel disease (IBD), the immunohistochemically visualized loss of claudin-3, the major sealing tight junction protein, from intestinal tissue resulted in the rapid appearance of this protein in the urine[19]. This was the first study to report that measurement of the status of tight junctions can be performed non-invasively (Figure 2).

Condition of enterocytes

Measurement of endogenous cytosolic enterocyte proteins in urine or plasma has been shown to be useful to estimate enterocyte damage. Fatty acid binding proteins (FABP) comprise a class of low molecular weight (14-15 kDa) cytosolic proteins found in high concentrations in tissues involved in the uptake and consumption of fatty acids. Three isoforms of FABP are present in the intestine: intestinal (I)-FABP, liver (L)-FABP, and ileal-bile acid binding protein (I-BABP)[20]. I-FABP, L-FABP, and I-BABP are in particular highly expressed in cells present on the tops of the villi. The presence of FABP on the tops of the villi, the initial site of destruction in numerous intestinal diseases, makes circulating FABP potentially useful plasma markers in early stages of intestinal diseases. The kidneys remove approximately 30% of FABP in a single pass, leading to a calculated FABP half-life of 11 min[21]. This emphasizes that FABP is an accurate marker for actual cell damage and that assessment of the urinary concentration is potentially useful in reflecting enterocyte damage, as FABP are rapidly cleared by the kidneys. Especially in neonates and children, this is a great advantage, as blood collection for diagnostic purposes is traumatic for children and a major cause for anaemia in neonates. Intestinal FABP (I-FABP) is primarily limited to mature enterocytes of the small and large intestine[20,22,23]. It circulates in low amounts in the blood stream of healthy individuals. I-FABP is a useful plasma/urinary marker for early enterocyte cell death and levels rise rapidly after episodes of acute intestinal ischaemia and inflamma-

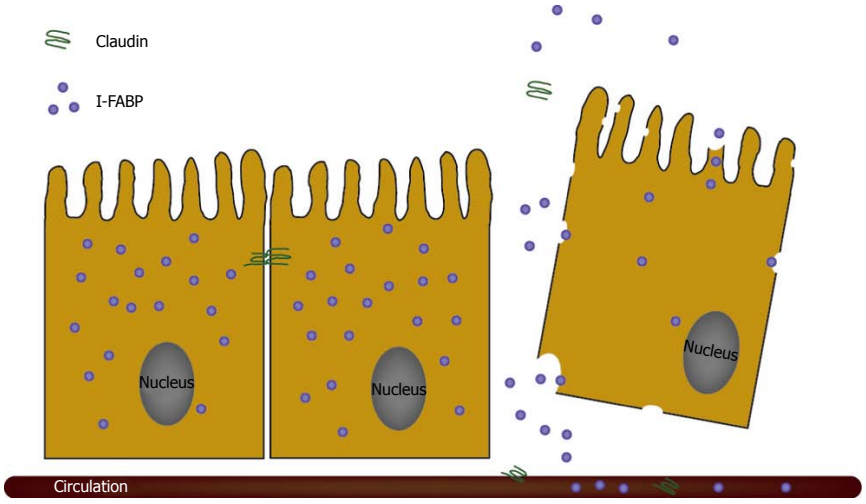

Figure 2 Leakage of fatty acid binding proteins and claudin, reflecting the condition of the epithelial layer of the gut.

tion[20,22,24,25] (Figure 2). The level of circulating I-FABP has been reported to correlate with the histological status of the epithelium after intestinal ischaemia-reperfusion in experimental studies[25,26]. A second gut-specific FABP is Ileal-Bile Acid Binding Protein (I-BABP), which is exclusively present in mature enterocytes of the jejunum and ileum[27]. Enterocytes also contain Liver-FABP (L-FABP), which is localised in the mature enterocytes of the small and large intestine, but is more abundantly present in the liver and to a lesser degree in tubular cells of the kidney[20,23]. Increased circulating and/or urinary L-FABP levels can therefore be derived from other organs than the intestine. Next to FABP, Glutathione S-Transferases (GST) are a family of more or less tissue specific cytosolic enzymes. These proteins are involved in the detoxification of xenobiotic compounds by conjugation to glutathione and grouped into species-dependent families based on their isoelectric point[28]. Alpha and pi GST are found in the small and large intestine[29]. However, these proteins are not organ-specific, as they are also expressed in liver and kidney[30]. Plasma levels of alpha GST are elevated upon ischaemic intestinal damage[31]. In conclusion, I-FABP and I-BABP are the most promising endogenous enterocyte proteins (markers) to assess enterocyte injury, as these proteins are specifically expressed in the gut and released immediately into the circulation upon cell damage.

IMMUNOLOGICAL COMPONENTS OF THE GUT WALL

Gut wall inflammation

A broad range of pathologies can lead to intestinal inflammation: neoplasia, IBD, infections, autoimmune diseases (e.g. celiac disease), ischaemia-reperfusion, intestinal hypoperfusion, and e.g. the use of non-steroidal anti-inflammatory drugs. Generally, defects or increased permeability of the mucosal barrier will cause intestinal inflammation in response to the enormous number of bacteria present in the bowel. Recruitment of leukocytes into the intestinal wall is important in the pathogenesis of intestinal inflammation[32]. Activated neutrophils infiltrate the mucosa and their products can be detected in faeces due to release into the intestinal lumen. Obviously, changes in neutrophil release products can also be detected in plasma/serum, but plasma/serum levels are also increased by various conditions other than gut inflammation. Therefore, faecal markers of neutrophils are specific for the detection of inflammatory intestinal diseases. Numerous neutrophil derived proteins present in stool have been studied, including calprotectin, lactoferrin, and elastase[32]. The most promising marker is calprotectin, because of its remarkable resistance to proteolytic degradation and its stability in stool kept at room temperature for at least seven days[33].

Calprotectin, also known as MRP-8/MRP-14 or S100A8/A9 complex, is a 36 kDa calcium and zinc binding heterodimer protein that plays a regulatory role in the inflammatory process. It constitutes about 60% of the soluble proteins in human neutrophilic cytosol and is also found in monocytes, macrophages, and ileal tissue eosinophils. It is released during cell activation or cell death and has antiproliferative, antimicrobial, and immunomodulating functions[32,33].

Faecal calprotectin is nowadays used in clinical practice to evaluate disease activity in the follow-up of patients treated for active IBD[32]. It was found that faecal calprotectin levels correlated well with endoscopic, as well as histological, disease activity of patients with IBD[32]. Moreover, recent studies showed that normalisation of calprotectin levels in patients with established IBD is a strong indicator of mucosal healing. Furthermore, several studies showed that calprotectin was a very sensitive detection marker of inflammation in patients with inflammatory bowel disease (IBD, Crohn's disease, and ulcerative colitis) compared with healthy controls and patients with irritable bowel syndrome, though not a specific marker, as increased levels were also found in neoplasia, infections, and polyps[32]. Bunn *et al*[34] confirmed these results on calprotectin levels in children with IBD. Carroll *et al*[35] stated that faecal calprotectin might be a useful marker of gastrointestinal mucosal inflammation in neonates in a pilot

study comparing seven patients with proven necrotising enterocolitis (NEC) with seven healthy peers. This is supported by our recent results[36].

CLINICAL IMPORTANCE OF GUT WALL INTEGRITY

Early diagnosis and follow-up of intestinal damage in clinical medicine

Evaluation of intestinal pathology in patients of all age groups has long been a challenge for clinicians. Numerous patients present with abdominal complaints, which are frequently aspecific and therefore correspond to pathologies of most intra- and even to some extra-abdominal organs. Laboratory tests and imaging techniques are often helpful in revealing disorders of organs, including the liver, pancreas, heart, and kidneys. However, it is still difficult to diagnose intestinal pathology in patients presenting with abdominal complaints[37]. The current standard technique for assessing intestinal status is endoscopy with biopsy. For some diseases this is helpful; however, it is invasive, associated with morbidity, sometimes requires sedation, expensive, and only assesses the function of the biopsied fraction. Moreover, for neutropenic and/or thrombocytopenic patients, the procedure is physically hazardous and often ethically unacceptable. Therefore, a major delay in diagnosis occurs in patients with e.g. NEC, chemotherapy-induced mucositis, acute mesenteric ischaemia, and celiac disease[37,38]. Such a diagnostic delay results in delayed treatment, which is accompanied by higher morbidity and mortality rates. In line with these diagnostic concerns, the follow-up of numerous intestinal diseases is hampered by the absence of rapid, non-invasive diagnostic means to assess intestinal damage for evaluation of the effects of treatment on the recovery of the disorder[37].

Potential involvement of the gut in the development of postoperative or posttraumatic complications

Patients undergoing major surgery or sustaining severe trauma are at risk of developing morbidity and mortality from post-operative or post-traumatic systemic inflammatory response syndrome (SIRS), sepsis, and multiple organ failure (MOF). Development of such potentially lethal complications in relatively healthy surgical or trauma patients is poorly understood[7]. Experimental animal studies have generated the hypothesis that the intestines are central in the origin of post-operative and post-traumatic sequelae[39-41]. Human studies have contributed insufficiently to gain insight in the applicability of this hypothesis[7]. Recognition of patients at risk of developing post-operative or post-traumatic SIRS, sepsis, and MOF is important, as patients with these clinical syndromes have the highest noncardiac mortality rate of patients in the intensive care unit (ICU)[39-42].

Experimental animal models, resembling the clinical situation of major surgery and trauma, show that haemorrhagic shock leads to disruption of the gut wall integrity, measured by derangement of tight-junctions and elevated circulating levels of FABP, originating from damaged intestinal epithelial cells[43,44]. Moreover, leakage of macromolecules, microbial products, and microbiota from the intestinal lumen into the circulation and mesenteric lymph nodes, spleen, and liver occurs[44]. The inflammatory response to microbial products, such as endotoxins, has been reported to be provoked *via* various rapidly induced innate immune mechanisms, ranging from Toll Like Receptors to complement activation. Supportive of the importance of the gut in the development of postoperative complications is the observation that protection of the gut wall integrity in hemorrhagically shocked animals by administration of probiotic strains, which inhibit the adhesion of enteric pathogens to intestinal epithelial cells, can result in abrogation of both local and systemic inflammatory responses[45]. However, administration of probiotic strains might also have unwanted side-effects, including disruption of intestinal tight junctions and increase of bacterial translocation[46].

Studies in patients undergoing major gastrointestinal, cardiac, or vascular surgery, investigating the role of the gut in the development of post-operative complications, are largely restricted to data on increased intestinal permeability for sugars, ^{51}Cr-ethylenediaminetetraacetic acid (^{51}Cr-EDTA), and the circulatory levels of endotoxin[14,47-49]. Increased intestinal absorption of sugars or ^{51}Cr-EDTA, indirect measures for gut barrier loss, in patients following major surgery support data obtained from animal studies, indicating that the gut barrier is injured after major surgery[14,48-50]. However, other reports show no changes in intestinal permeability[51]. Moreover, the value of measuring gut integrity with sugar probes is debatable[8,9]. Similar results are reported on the development of endotoxemia. Several authors report on increased circulating levels of endotoxin or reduced values of anti-endotoxin antibody during major surgery, suggesting leakage of gut derived bacterial products from the gut lumen into the circulation due to an impaired epithelial barrier, whereas others show unaltered endotoxin concentrations[49,52]. Data showing better survival in patients with pre-operatively higher anti-endotoxin titres support the theory of intestinal contribution in the development of postoperative complications[53]. In conclusion, the debate regarding involvement of the gut in patients undergoing major surgery is still ongoing.

Three recent studies show the temporary presence of intestinal villous cell damage, measured by increased urinary levels of I-FABP, in patients undergoing cardiovascular surgery with cardiopulmonary bypass (CPB)[54-56]. Patients with high urinary I-FABP levels developed postoperative gastro-intestinal complications[54]. The use of CPB was shown to be responsible for alterations in blood flow with consequent intestinal mucosal hypoxia and villous tip ischaemia[55,56]. Furthermore, intestinal injury correlated positively with markers of systemic inflammation. We recently reported, in patients undergoing major non-abdominal surgery, a similar influence of reduction in blood pressure on the provocation of intestinal villous cell

injury, without the use of extracorporeal circulation[57]. A significant inverse association between mean arterial pressure and succeeding plasma levels of I-FABP and I-BABP was found, which indicates that systemic hypotension is correlated with intestinal mucosal cell injury. Anaesthetics, leading to decreased systemic vascular resistance, mainly cause systemic hypotension. Finally, splanchnic hypoperfusion (measured by gastric mucosal $PiCO_2$ and $P_{r-a}CO_2$-gap) correlated strongly with intestinal mucosal damage (assessed by plasma I-FABP) at all observed time-points during surgery[57].

In conclusion, the results of these studies show, for the first time, the relation between altered splanchnic perfusion and the development of intestinal mucosal cellular damage in patients undergoing major surgery. Collectively, these findings shed new light on the potential role of intestinal barrier compromise during major surgery, which was deduced from numerous animal studies, but has now been reported, for the first time, in relatively healthy children and adolescents undergoing major (non-abdominal) surgery. Furthermore, these results indicate a need to re-examine currently accepted criteria of haemodynamic parameters, both regarding the use of extra-corporeal circulation and accepted systemic hypotension, in patients undergoing major surgery.

The presence of intestinal damage does not show any cause-and-effect relationship with the development of sepsis or MOF. Moreover, intestinal damage may be part of more generalised tissue damage with epithelial barrier dysfunction in lung, liver, and kidney[7]. These studies are, however, the basis for further research to clarify the onset of intestinal damage. In addition, assessment of intestinal epithelial damage in patients with sepsis or undergoing major surgery might have important clinical implications. Evaluation of intestinal epithelial cell damage as a consequence of splanchnic hypoperfusion, could help to monitor preoperative treatment or therapy in patients with shock directed at restoration of peripheral perfusion and prevention of organ damage.

FUTURE DIRECTIONS

Relatively new markers for gut wall integrity (including FABP, claudin-3, and calprotectin) might be useful in the early diagnosis of intestinal diseases. The implementation of these markers in daily clinical practice seems only a matter of time. In addition, these markers are used nowadays to unravel the involvement of the gut in patients undergoing major surgery or sustaining major trauma or sepsis. These results led to studies aimed at re-examining accepted criteria of preoperative haemodynamic parameters, both regarding the use of extra-corporeal circulation and accepted systemic hypotension in patients undergoing major surgery[56,57]. We strongly believe that I-FABP can be used as a clinical marker of intestinal hypoperfusion. First of all, there is a strong correlation between gastric mucosal $PiCO_2$ and circulating levels of I-FABP, in patients with postoperative sepsis who were admitted to the ICU[42]. Splanchnic hypoperfusion in the early phase of abdominal sepsis correlated strongly with intestinal mucosal damage. Moreover, elevated plasma I-FABP values on admission to the ICU were associated with a poor outcome in patients with abdominal sepsis. In children with meningococcal sepsis, it was shown that almost half of the patients presented with intestinal epithelial cell damage, shown by increased plasma I-FABP values, at admission to the paediatric ICU. The children who died were characterised by continued presence of gut damage, while in all survivors this injury came to an end within 12 h after starting intensive treatment[58]. A significant proportion (93%) of adult trauma patients rapidly developed intestinal mucosal cell damage, measured by elevation of plasma I-FABP values[59]. The extent of intestinal damage was readily detectable in blood withdrawn on presentation at the emergency department (ER). Interestingly, the highest 10% of I-FABP values at ER belonged to patients with severe abdominal trauma that required acute surgical intervention, such as ruptures of the diaphragm, liver, and spleen. Circulatory concentrations of enterocyte damage marker I-FABP were related to the presence of shock and the extent of general injury, as well as abdominal trauma, indicating that the level of intestinal cell damage was determined by both systemic and local factors. Moreover, early I-FABP values correlated positively with the inflammatory response that developed in the days following trauma[59].

In conclusion, evaluation of intestinal tissue damage in the early phase of sepsis is an adequate predictor for survival. Furthermore, the adequacy of treatment of circulatory failure in sepsis is currently monitored using indirect parameters of (peripheral) tissue perfusion and oxygenation, including blood pressure, urinary output, metabolic status (lactate, base deficit) skin temperature, $ScvO_2$, and capillary refill time. However, these parameters do not reflect the actual defects in (peripheral) tissue perfusion and subsequent tissue damage. Assessment of plasma I-FABP levels offers the possibility to monitor the presence of intestinal epithelial cell damage as a consequence of splanchnic hypoperfusion, which could help to monitor treatment directed at restoration of peripheral perfusion and prevention of organ damage. Further studies are needed to clarify the diagnostic potential of assessment of plasma I-FABP in monitoring the treatment of sepsis in the acute phase and during follow-up.

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TOPIC HIGHLIGHT

Yoshihisa Takahashi, MD, *Series Editor*

Pediatric nonalcoholic fatty liver disease: Overview with emphasis on histology

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is a disease in which excessive fat accumulates in the liver of a patient without a history of alcohol abuse. This disease includes simple steatosis and nonalcoholic steatohepatitis (NASH). NAFLD/NASH is recognized as a hepatic manifestation of metabolic syndrome. In recent years, pediatric NAFLD has increased in line with the increased prevalence of pediatric obesity. The estimated prevalence of pediatric NAFLD is 2.6%-9.6%, and it is associated with sex, age, and ethnicity. With regard to the pathogenesis of NAFLD, the "two-hit" hypothesis is widely accepted and oxidative stress is thought to play an important role in the second hit. Although clinical symptoms, laboratory data, and imaging findings are important, liver biopsy is regarded as the gold standard for the diagnosis of NAFLD/NASH. In addition, liver biopsy is essential for assessing the degree of necro-inflammatory change and fibrosis in NASH. Two different types of steatohepatitis (type 1 and type 2 NASH) have been reported, with type 2 NASH being present in as many as 51% of pediatric NAFLD patients. However, we and others have observed that type 1 and 2 patterns commonly overlap. Although pharmacotherapy has been studied in clinical trials, lifestyle modification by diet and exercise remains the mainstay of treatment for NAFLD/NASH.

Key words: Children; Histology; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Obesity

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a disease in which excessive fat accumulates in the liver of a patient without a history of alcohol abuse. There are two types of NAFLD: simple steatosis, demonstrating only fat deposition in hepatocytes, and nonalcoholic steatohepatitis (NASH), demonstrating not only steatosis but also a necro-inflammatory reaction. NASH can progress to liver cirrhosis and result in complications that include hepatocellular carcinoma[1]. NAFLD/NASH is recognized as a hepatic manifestation of metabolic syndrome[2], and elements of metabolic syndrome, such as central obesity, type 2 diabetes, hypertension, and hypertriglyceridemia, are well-known risk factors for NAFLD[3-5].

In recent years, pediatric NAFLD has increased in line with the increased prevalence of pediatric obesity and has become an important worldwide health problem. Pediatric NAFLD has different histological characteristics

from those of adult NAFLD[6]. In this article, we outline the epidemiology, pathogenesis, diagnosis, histological features, and treatment of pediatric NAFLD.

EPIDEMIOLOGY

Approximately 20% of adults are estimated to have NAFLD, and 2%-3% of adults have NASH[7]. On the other hand, in children and adolescents, researchers from America[8] and Asia[9,10] estimate that the prevalence of NAFLD is 2.6%-9.6%. These data, however, must be interpreted carefully because the diagnostic criteria of NAFLD adopted in these studies are diverse and the accurate diagnosis of NAFLD and NASH requires histological evaluation, which is impossible to obtain for all subjects in population-based studies. Studies from Europe[11-13], Asia[14-16], and America[8] estimate that the prevalence of NAFLD among overweight or obese children and adolescents is 24%-77%. Despite the diversity of diagnostic criteria used in these studies, obesity is thought to be an important risk factor for pediatric NAFLD.

NAFLD is consistently more prevalent in boys than in girls[17] suggesting that sex hormones are associated with the occurrence of pediatric NAFLD. NAFLD is more prevalent in adolescents than in younger children[8] and sex hormones and insulin resistance in puberty may be the cause of this. The rate of NAFLD in African American children is lower than in Hispanic and Caucasian children, despite African American children being prone to having risk factors for NAFLD, such as obesity, insulin resistance, and diabetes[17].

PATHOGENESIS

The "two-hit" hypothesis proposed by Day *et al*[18] is widely accepted as the pathogenesis of NAFLD/NASH; the first hit causes fat accumulation in hepatocytes, and the second hit causes inflammation and fibrosis. Fat accumulation in the liver is closely associated with metabolic derangements that are related to central obesity and insulin resistance. Fat accumulation can be caused by the increased delivery of free fatty acids to the liver, disordered metabolism of fatty acids by hepatocytes, or increased *de novo* synthesis of fatty acids and triglycerides[19-21].

Oxidative stress is thought to play an important role in the second hit by causing peroxidation of lipids that accumulate in hepatocytes. The cause of oxidative stress is the overproduction of reactive oxygen species (ROS) by induction of CYP 2E1 in the liver, mitochondrial dysfunction, and so on[22,23]. Peroxidation of lipids causes mitochondrial dysfunction and results in the overproduction of ROS, thus forming a vicious cycle[24-27]. The immune response toward lipid peroxidation products has been associated with the progression of NAFLD[28]. Other changes that can mediate liver inflammation include an increase in inflammatory cytokines such as tumor necrosis factor-α and a decrease in anti-inflammatory cytokines such as adiponectin[2,29].

DIAGNOSIS

To diagnose NAFLD, the exclusion of other liver diseases such as hepatitis B and C, autoimmune hepatitis, drug-induced liver injury, Wilson's disease, and α1-antitrypsin deficiency is necessary. Pediatric NAFLD is often asymptomatic; however, patients may occasionally complain of abdominal pain, fatigue, or malaise[30-32]. Hepatomegaly is often observed on physical examination, although this finding may be missed in clinical practice[33]. Acanthosis nigricans (black pigmentation at skin folds or axillae that is associated with hyperinsulinemia) has been reported in up to 50% of pediatric NASH cases[30,34]. A family history of NAFLD should be referred to because familial clustering is often detected[35]. Increased serum levels of aspartate aminotransferase and alanine aminotransferase (ALT) are predictors of the existence of NAFLD/NASH; however, normal serum levels of these aminotransferases do not exclude the existence of NAFLD/NASH. Although an elevated ALT and/or an enlarged echogenic liver, as revealed by ultrasonography, in the setting of overweight or obesity and/or evidence of insulin resistance are highly suggestive of NAFLD, histological evaluation remains the only means of accurately assessing the degree of steatosis, necro-inflammatory change, and fibrosis found in NASH and in distinguishing NASH from simple steatosis[36]. In addition, liver biopsy is useful in differentiating NAFLD from other liver diseases. Because pediatric NAFLD has different histological characteristics compared to adult NAFLD, careful histological evaluation is necessary.

Liver biopsy is regarded as the gold standard for the diagnosis of NAFLD/NASH; however, this procedure has some problems, such as high risk, high cost, and sampling error. Therefore, it is necessary to develop improved noninvasive methods for accurate screening and diagnosis. Currently, ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) are the imaging modalities generally used for the evaluation of hepatic steatosis. Although ultrasonography has some merits such as widespread availability, lack of radiation, and relatively low cost, it also has some demerits such as operator dependence, subjectivity in interpretation, unclear images in severely obese patients, and inability to detect mild hepatic steatosis. CT is not favored for use in children because it uses ionizing radiation. MRI appears to be a more accurate and reproducible method[37]. In addition, because MRI is not subject to interpretation or interobserver variation, it may be better than ultrasound for quantifying hepatic fat in children[38-40]. However, neither CT nor MRI can judge low-grade fibrosis. Ultrasound elastography and magnetic resonance elastography have been developed as modalities to measure liver stiffness which is a surrogate marker of liver fibrosis. Investigations are needed to determine the usefulness of these modalities in diagnosing pediatric NAFLD.

HISTOLOGY

The histological characteristics of pediatric NAFLD/

NASH differ from those of adult NAFLD/NASH. Schwimmer *et al*[6] examined the histological appearance of 100 pediatric NAFLD cases and identified two different types of steatohepatitis. Type 1 NASH was consistent with NASH as described in adults and was characterized by steatosis, ballooning degeneration, and/or perisinusoidal fibrosis in the absence of portal changes. Type 2 NASH was a peculiar histological pattern in children and was characterized by steatosis, portal inflammation, and/or portal fibrosis in the absence of ballooning degeneration and perisinusoidal fibrosis. Type 1 NASH was reported to be present in only 17% of pediatric NAFLD, whereas type 2 NASH was present in 51%. Simple steatosis was present in 16% of subjects. Advanced fibrosis was present in 8% and liver cirrhosis was present in 3%. In cases of advanced fibrosis, the pattern was generally that of type 2 NASH. Children with type 2 NASH were significantly younger and had a greater severity of obesity than children with type 1 NASH. Boys were significantly more likely to have type 2 NASH and less likely to have type 1 NASH than girls. Type 2 NASH was more common in children of Asian, Native American, and Hispanic ethnicity. Type 1 and type 2 NASH may have a different pathogenesis, natural history, and response to treatment. It is currently unclear whether type 2 NASH changes to type 1 NASH as the patient ages.

Subsequently, Nobili *et al*[41] reported that type 1 NASH was present in only 2% of their 84 pediatric NAFLD subjects and type 2 NASH was present in 29%. The combination of both types was present in 52% of their pediatric NAFLD subjects, and simple steatosis was present in 17%. Accordingly, the overlap of type 1 and 2 was the most common pattern in their study. The discrepancy between the results of these studies may reflect the different ethnicity of subjects, which were mainly Hispanics and Asians in the study by Schwimmer *et al*[6] and were all Caucasians in the study by Nobili *et al*[41].

We compared the histological appearance of needle biopsy specimens from 34 pediatric and 23 adult NAFLD cases in Japan (unpublished data) and found that steatosis tended to be more severe in children as compared to adults (Figure 1A). Perisinusoidal fibrosis was significantly less frequent in children than in adults and intralobular inflammation and ballooning degeneration tended to be milder in children as compared to adults. Although portal inflammation tended to be more severe in children than in adults (Figure 1B), there was no obvious difference in the degree of portal fibrosis between the two groups. Type 2 NASH was present in 21% of pediatric subjects and in 9% of adult subjects. Accordingly, we confirmed that pediatric NAFLD had different histological characteristics from those of adult NAFLD in Japanese patients also. However, we found that the prevalence of type 2 NASH in pediatric subjects was less than that in the study by Schwimmer *et al*[6] and that the overlap of type 1 and 2 patterns was common as noted by Nobili *et al*[41].

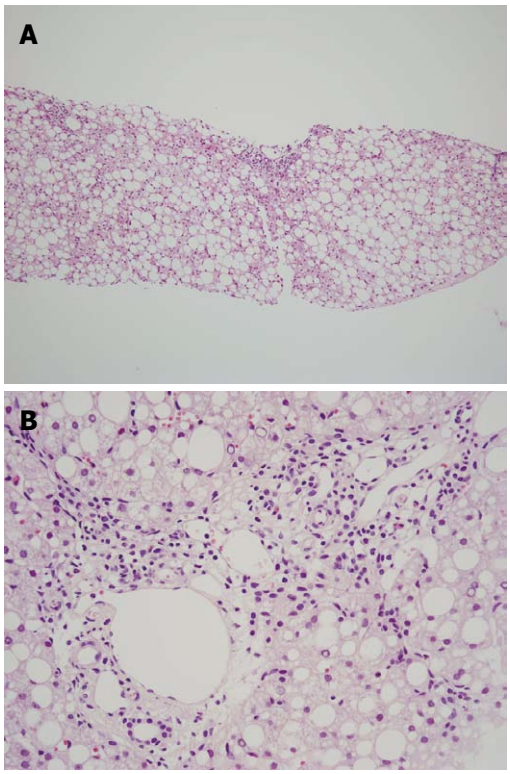

Figure 1 Histological appearance of a case of pediatric nonalcoholic fatty liver disease. A: Marked steatosis with panlobular distribution is observed (HE stain, × 40); B: Moderate inflammation is observed in the portal area (HE stain, × 200).

SPECULATED FACTORS THAT SEPARATE PEDIATRIC NAFLD AND ADULT NAFLD

The abovementioned histological differences between pediatric and adult NAFLD might reflect different etiological factors between them. In a study of morbidly obese adults undergoing bariatric surgery, portal inflammation was reported in 24%[42]. In a study of adult NAFLD subjects, which was composed of mainly male patients, portal inflammation and portal fibrosis were observed in 63% and 6% of patients, respectively[43]. Thus, it is conceivable that adult NAFLD patients, especially severely obese patients and male patients, may also show a pediatric-type histological pattern. As mentioned above, NAFLD is consistently more prevalent in boys than in girls[17], and type 2 NASH was more common in severely obese children and boys in the study by Schwimmer *et al*[6]. Accordingly, obesity and sex hormones might be critical etiological factors in pediatric NAFLD. In our histological investigation, steatosis tended to be more severe in pediatric NAFLD than in adult NAFLD, and its distribution was not restricted to zone 3 in many pediatric cases (unpublished data). Recently, we found that administration of fructose- or sucrose-enriched diet to Wistar rats induces hepatic steatosis that is mainly dis-

tributed in zone 1 (unpublished data). It is conceivable that inappropriate food consumption, especially excessive drinking of sweet beverages, might be an important cause of pediatric NAFLD; thus, the rat model might be a good model of pediatric NAFLD.

TREATMENT

Obesity, hyperlipemia, insulin resistance, and hyperglycemia are well-known risk factors for NAFLD and oxidative stress is thought to be important in the progression from simple steatosis to NASH. Reducing the risk from these factors is considered to be an effective treatment for NAFLD. The main targets of therapy are normalization of the serum ALT level and improvement in histopathology as shown by biopsy. Lifestyle change (diet and exercise) and pharmacotherapy have been studied.

Diet and exercise

Most children with NAFLD are overweight or obese, and weight loss is thought to be an effective preventative measure and/or treatment for pediatric NAFLD. In fact, lifestyle modification remains the mainstay of treatment for NAFLD of obese children. Gradual weight loss by diet and proper exercise has been shown to improve serum aminotransferase levels and liver histology of adult patients with NAFLD[44-46]. In a study of pediatric NAFLD subjects, moderate weight loss by lifestyle intervention lowered serum aminotransferase levels and improved hepatic steatosis, lobular inflammation, and hepatocyte ballooning; however, the degree of fibrosis did not change[47]. Based on the pathogenesis of NAFLD, an appropriate diet for NAFLD treatment is thought to be a low-glycemic index diet. Very rapid weight loss by excessive caloric restriction is not recommended because it may increase dysmetabolism and liver inflammation and fibrosis[48].

Vitamin E

Because oxidative stress plays a pivotal role in the pathogenesis of NASH, administration of antioxidants, such as vitamin E, is expected to be an effective therapy for NASH. In an open-label study, a 2-4 mo treatment with vitamin E (400-1200 IU/d orally) normalized serum aminotransferase levels in obese children with NASH but did not improve steatosis on ultrasonography, and serum aminotransferase returned to abnormal levels when the treatment was stopped[49].

Insulin sensitizers

Most children with NAFLD have insulin resistance, and therefore, insulin sensitizers may be an effective treatment for this disease. Metformin has been used successfully for improving insulin resistance and possibly liver histology in adults[50,51]. In addition, the efficacy and safety of metformin in the treatment of pediatric type 2 diabetes has been confirmed[52,53]. Open-label treatment with metformin for 24 wk showed notable improvement in liver chemistry, liver fat, insulin sensitivity, and quality of life in pediatric NASH patients[54]. Pioglitazone has been reported to improve biochemical and histological features of adult NASH[55,56]; however, data on the safety of pioglitazone in children are currently insufficient, and future investigations are needed in order to use this drug in pediatric NAFLD.

Ursodeoxycholic acid

Ursodeoxycholic acid (UDCA) is a hydrophilic bile acid that operates as an antioxidative and cytoprotective agent. A randomized control trial (RCT) involving 31 obese children with abnormal serum aminotransferase levels, found that UDCA (10 mg/kg per day) was ineffective, both alone and when combined with diet, in reducing serum aminotransferases or the appearance of steatosis on ultrasonography[57]. Similarly, in two RCTs involving adult NAFLD/NASH, UDCA did not exert effects on liver chemistry or histology[58,59]. However, in one RCT, UDCA in combination with vitamin E improved serum aminotransferases and hepatic steatosis in NASH patients[60].

Bariatric surgery

In 2007, Furuya *et al*[61] examined the histological changes in the liver after Roux-en-Y gastric bypass surgery performed in 18 severely obese NAFLD patients. As a result, steatosis disappeared in 84%, fibrosis disappeared in 75%, and hepatocellular ballooning disappeared in 50% of patients after 2 years. These improvements were likely a function of the substantial weight loss. Although the role of bariatric surgery for NAFLD in severely obese adolescents has not been studied, this treatment may be promising.

CONCLUSION

Pediatric NAFLD has been increasing worldwide and this tendency will continue as long as pediatric obesity increases. Liver biopsy is the gold standard for the diagnosis of NAFLD; however, advances in noninvasive tests are expected. Pediatric NAFLD has different histological characteristics compared to adult NAFLD. Diet and exercise are the mainstays of treatment in NAFLD; however, advances in pharmacotherapy are expected.

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TOPIC HIGHLIGHT

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Histopathology of nonalcoholic fatty liver disease

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Abstract

Histological analysis of liver biopsies remains a standard against which other methods of assessment for the presence and amount of hepatic injury due to nonalcoholic fatty liver disease (NAFLD) are measured. Histological evaluation remains the sole method of distinguishing steatosis from advanced forms of NAFLD, i.e. nonalcoholic steatohepatitis (NASH) and fibrosis. Included in the lesions of NAFLD are steatosis, lobular and portal inflammation, hepatocyte injury in the forms of ballooning and apoptosis, and fibrosis. However, patterns of these lesions are as distinguishing as the lesions themselves. Liver injury in adults and children due to NAFLD may have different histological patterns. In this review, the rationale for liver biopsy, as well as the histopathological lesions, the microscopically observable patterns of injury, and the differential diagnoses of NAFLD and NASH are discussed.

Key words: Fatty liver; Steatosis; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Fibrosis

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INTRODUCTION

Liver biopsy evaluation was the foundation for the proposals that certain individuals who do not consume excess alcohol could, in fact, suffer from a form of chronic progressive liver disease characterized by lesions that had been considered those of alcohol, namely, steatosis, ballooned hepatocytes with Mallory-Denk hyaline bodies, and the particular form of fibrosis of alcohol. Several authors had been evaluating this concept[1-5], when the seminal manuscript of Ludwig *et al*[6] was published in 1980 that described 20 subjects. Since that time, much of the progress that has been made in studies of natural history[7-12], clinical associations of histological assessments of activity and fibrosis, correlations with metabolic syndrome[13,14] and serum markers of cardio-vascular disease[15], the variability of ethnic susceptibility[16-18], and the presence of hepatocellular carcinoma in cirrhotics and non-cirrhotics with nonalcoholic fatty liver disease (NAFLD)[19-21] have occurred by evaluating liver biopsies.

The clinical and scientific needs for imaging studies and serum-based assays to "predict" the presence of steatohepatitis, fibrosis, and/or inflammation are based on a well-recognized concept that liver biopsy is invasive, potentially harmful, and may suffer from "sampling error"[22,23]. Furthermore, liver biopsy cannot be considered

a screening tool for population studies. However, it is also recognized that, imperfect as it is, liver biopsy evaluation remains the standard against which other assays and clinical algorithms must be matched, and prospectively validated.

Liver biopsy does, indeed, suffer from challenges. Many can be overcome by the realization that even an adequate biopsy represents only 1/50 000-1/65 000 of this large organ. Therefore, high quality biopsy techniques, such as utilization of appropriately sized needles[24], careful choice of sampled area, and appropriate tissue preparation and interpretation are all important considerations for liver biopsy. Pathologists recognize that wedge biopsies are inferior to appropriately-sized and placed needle core biopsies for evaluation of a diffuse parenchymal disease. In addition, there might be differences between right and left lobes of the liver, as the left lobe is relatively smaller, is covered by more capsule per unit area, and the subcapsular portal tracts can be deceptively more fibrous than those of deeper parenchyma. Differences in histopathological findings have been demonstrated in studies of bariatric patients biopsied concurrently from the right and left lobes[25]; these differences were abrogated somewhat by the use of a large bore needle in another study[24]. Adequate sample length has also been noted as a correlative factor with histological evaluation in NAFLD[26], with biopsies $\geqslant$ 1.5 cm long having a higher yield of definitive nonalcoholic steatohepatitis (NASH) diagnosis compared to those measuring < 1 cm in length (29% *vs* 56%)[26]. Small biopsies (< 1.6 cm) show higher variability for NAFLD fibrosis stage than longer biopsies[27]. It has been proposed that sampling error might be reduced by using larger gauge needles[24], obtaining longer ($\geqslant$ 1.5 cm) biopsies[28], or by taking more than one tissue core when possible[26]. Once in the laboratory, liver biopsies should not be prepared on tissue "sponges", as these create space-distorting artifacts[29]. Of at least equal, if not greater, significance, however, is pathologists' training and focus in the field. Just as one doesn't ask a non-specialized surgeon to perform cardiac bypass surgery, one should also recognize the need for trained liver pathologists. Studies in chronic hepatitis C (CHC) liver biopsy evaluation that compared the expertise of liver pathologists in academic practice with community pathologists showed this to be a potential source of discordance in histological evaluation[30]. The number of independent readings has also been shown to correlate with greater yield of findings[26].

VALUE OF LIVER BIOPSY EVALUATION IN NONALCOHOLIC FATTY LIVER DISEASE

Pro's and Con's of liver biopsy

The large series of over 350 liver biopsies done for clinically unexplained liver test elevation in adults by Skelly *et al*[31] highlights the value of liver biopsy evaluation in this setting. While two-thirds of the biopsies showed some form of nonalcoholic fatty liver disease, the remainder were either "normal" (6%) or had histopathological features of subsequently clinically confirmed liver disease, such as hereditary hemochromatosis, drug-induced liver injury, and autoimmune liver disease. Several series have now shown the lack of correlation of alanine aminotransferase (ALT) levels and both necroinflammatory activity and fibrosis, including cirrhosis, in subjects with known NAFLD[32-34], including obese children[35]. Liver biopsy evaluation is also important for a patient with a clinical suspicion of autoimmune liver disease. Up to 20% of NAFLD have positive serologies for antinuclear antibody, antismooth muscle antibody, and/or antimitochondrial antibody[36-38]; therefore, liver biopsy evaluation might be the only means of determining the underlying cause of the disease. Furthermore, surgeons have shown that intraoperative visualization is commonly not correct for the prediction of either steatosis or advanced fibrosis, and thus liver biopsy is currently being recommended in the morbidly obese bariatric population at the time of surgery[39].

The final "pro" of liver biopsy is both confirmation of the diagnosis, as well as evaluation and semiquantitation of necroinflammatory lesions and fibrosis, and evaluation of architectural remodeling. To date, noninvasive tests have shown to be effective for the extremes: either lack of, or abundance of, inflammation or fibrosis, but overall sensitivity and specificity for the lesions in between are less impressive[22]. A recently studied modification of the European Liver Fibrosis score, named the Enhanced Liver Fibrosis score, has shown the most promise in terms of predicting over 75% of adult patients who would not need a liver biopsy, regardless of stage[40]. Measurement of serum keratin 18 (K18) fragments, a marker of hepatocyte apoptosis, has emerged as a promising biomarker for NASH, with sensitivity and specificity of up to 77% and 92%, respectively[41]. Transient elastography measuring liver stiffness has been recently shown to be a useful noninvasive test to exclude advanced fibrosis in Chinese NAFLD patients, with a negative predictive value of 97%[42]. Limitations and need for further testing of this modality are reviewed[43].

The arguments against liver biopsy in NAFLD include the invasive nature of the procedure, the lack of effective treatments beyond recommendations for dietary changes, weight loss and exercise, the fact that only a minority of NAFLD patients have the progressive lesions of NASH[44], and the known improvements of diagnosis with clinically-derived tests[45,46] and imaging techniques[47]. However, at present, the final correlation for any of the new tests is liver biopsy evaluation.

HISTOLOGY OF NAFLD IN ADULTS

The histological spectrum of NAFLD includes various forms of small and large droplet macrovesicular steatosis, with or without lobular and portal inflammation, and steatohepatitis, which is characterized by steatosis, inflammation, and cell injury, i.e. NASH. Progression of fibrosis and architectural remodeling is thought to develop in 10%-15% of NASH patients, and cirrhosis

in 15%-25%. Thus, of all individuals with some form(s) of fatty liver, 3%-5% may develop cirrhosis[48]. Cirrhosis due to NASH and cryptogenic cirrhosis might both result in hepatocellular carcinoma (HCC)[19,49]. Rarely, HCC may occur in non-cirrhotic NASH[21,50].

NAFLD

The main histological characteristic of NAFLD, as its name implies, is the accumulation of fat in the form of triglycerides within hepatocytes, a lesion originally termed "steatosis" after the ancient Greek word for fat, "stear". The presence of > 5% steatotic hepatocytes in a liver tissue section is now accepted as the minimum criterion for the histological diagnosis of NAFLD[51,52].

Steatosis in NAFLD is usually macrovesicular, referring to hepatocytes with a single large intracytoplasmic fat droplet or smaller well defined droplets displacing the nucleus to the cell periphery. Mixed steatosis might also occur, when, in addition to macrovesicular steatosis, groups of hepatocytes with centrally placed nuclei and numerous minute lipid droplets in the cytoplasm are observed. Pure microvesicular steatosis, needing special stains such as Oil-Red-O to identify the intracytoplasmic material as fat, is not a common feature of NAFLD; however, small, azonal collections of hepatocytes might have this type of fat. In simple NAFLD, in addition to steatosis, foci of lobular inflammation, mild portal inflammation, and lipogranulomas may be seen. However, features of hepatocellular injury and fibrosis, indicating progression to steatohepatitis, are not observed, by definition[51].

The extent of steatosis is commonly evaluated and reported semi-quantitatively. The most reproducible method follows the acinar architecture dividing the liver parenchyma in thirds and assessing percentage involvement by steatotic hepatocytes: 0%-33% (or 0%-5%, 5%-33%)-mild, 33%-66%-moderate or > 66% - severe steatosis[53,54].

One of the distinctive features of steatosis in adult NAFLD, in contrast to most pediatric NAFLD cases, is its predilection to start in acinar zone 3 (perivenular). When steatosis is severe it can occupy the whole acinus. Steatosis might not persist during the progression of fibrosis to remodeling and cirrhosis; thus, steatosis might not be reliably identified in a cirrhotic liver[55].

Adult NASH

Most hepatopathologists agree that the minimal criteria for the histological diagnosis of adult NASH include steatosis, hepatocyte injury, usually in the form of ballooning, and lobular inflammation, typically localized in acinar zone 3. Fibrosis, as in other forms of chronic hepatitis, is not a required diagnostic feature of NASH[56-58].

Hepatocellular injury in NASH can take the form of ballooning, apoptosis, or lytic necrosis[51]. Ballooning is a feature of major importance in NASH as its presence has been associated in prognostic studies with more aggressive disease and high incidence of cirrhosis[59]. Furthermore, the presence of ballooned hepatocytes on liver biopsy has been linked with features of the metabolic syndrome[60]. Ballooned hepatocytes are enlarged, with swollen, rarefied, pale cytoplasm and, usually, show a large, hyperchromatic nucleus, often with a prominent nucleolus. They might be the result of alterations in intermediate filament cytoskeleton[61] and/or presence of microvesicular steatosis[62]. The histological recognition of hepatocellular ballooning can show significant inter-observer variation[63]. Loss of the normal hepatocyte keratins, 8 and 18, as detected by immunostaining, might help in the objective identification of ballooned hepatocytes[64].

Apoptotic (acidophil) bodies, another form of hepatocyte injury and a feature of programmed cell death, are common in NASH[65]. They can easily be identified on routine stains, but are further highlighted by immunohistochemistry for keratin 18 fragments, the same antigen that has been recently proposed as a biomarker of NASH[41,66]. The number of acidophil bodies per mm^2 of liver tissue (acidophil body index) has been proposed to serve as a complementary histological feature when diagnosis of NASH is uncertain[67].

Lobular inflammation is usually mild, and consists of a mixed inflammatory cell infiltrate, composed of lymphocytes, some eosinophils, and, occasionally, a few neutrophils. Polymorphs are occasionally observed surrounding ballooned hepatocytes in a lesion known as "satellitosis". Foci of chronic lobular inflammation, consisting mainly of lymphocytes, are occasionally seen. Scattered lobular microgranulomas (Kupffer cell aggregates) and lipogranulomas are common[57,68].

Portal chronic mononuclear cell inflammation in adult NASH is not uncommon and is usually mild. When severe or disproportionate to the acinar lesions, the possibility of concurrent CHC should be excluded[56,68,69]. In untreated NAFLD patients, increased portal inflammation has been proposed as a marker of severe disease[70], and has been correlated with the diagnosis of definite steatohepatitis[71].

Fibrosis in adult NASH usually starts in acinar zone 3 and has a characteristic "chicken wire" pattern due to deposition of collagen and other extracellular matrix fibers along the sinusoids of zone 3 and around the hepatocytes. Portal fibrosis without perisinusoidal/pericellular fibrosis has been reported in some cases of morbid obesity-related NASH[72,73] and in pediatric NASH (discussed below). In advanced disease, bridging fibrosis and cirrhosis might develop[51]. NASH-related cirrhosis is most commonly macronodular or mixed. At the cirrhotic stage, perisinusoidal fibrosis and other features of "active" disease may or may not be evident. Therefore, in the absence of a previous biopsy with NASH or other disease-specific histology, the cirrhosis may be labeled "cryptogenic". In a recent meta-analysis of ten longitudinal histological studies of NASH, presence of inflammation in the initial biopsy and age emerged as independent predictors of progression to advanced fibrosis in patients with NASH[74].

Sinusoidal collagen formation in NASH, as in other chronic liver diseases, is likely to be the result of hepatic stellate cell (HSC) activation[75,76]. The HSC activation score, as measured by alpha-smooth muscle actin immunohistochemistry, was shown to predict progression of fibrosis in NAFLD[77]. Portal fibrosis, on the other

hand, has been linked to the ductular reaction (i.e. ductular proliferation at the portal tract interface arising from progenitor cells in the periportal area and accompanied by neutrophils and stromal changes). These findings have been correlated with insulin resistance, impaired hepatocellular replication, and advanced stages of fibrosis, indicating that it might provide a pathway for progressive fibrosis[78]. Recent work has found evidence of epithelial-to-mesenchymal transition of ductular cells *via* activation of the hedgehog pathway, a process by which mature epithelial cells differentiate into cells with a mesenchymal phenotype and function, in NAFLD[79].

Other histological lesions that may be seen in NASH include Mallory-Denk bodies (MDB), megamitochondria, glycogenated nuclei, and iron deposition. MDB (previously called Mallory bodies or Mallory's hyaline)[61] are eosinophilic intracytoplasmic inclusions commonly seen close to the nucleus of ballooned hepatocytes in zone 3, usually in areas of perisinusoidal fibrosis. They are composed of misfolded intermediate filaments (keratins 8 and 18), ubiquitin, heat shock proteins, and p62[80]. MDB have been correlated with increased necroinflammatory activity[53] and with a higher incidence of cirrhosis[59]. Although not a requirement for the histological identification of NASH, the presence of MDB strengthens the diagnosis. MDB are not, however, restricted to NASH and can be seen in steatohepatitis due to other causes (alcoholic, drug-induced, etc), as well as in chronic cholestasis, metabolic diseases, and hepatocellular neoplasms[81].

Megamitochondria (giant mitochondria) are round or needle-shaped, eosinophilic, intracytoplasmic inclusions more commonly observed in hepatocytes with microvesicular steatosis. The are not zonally restricted[82]. Ultrastructural studies have shown that these abnormal mitochondria show loss of cristae, multilamellar membranes, and paracrystalline inclusions[82,83]. Megamitochondria in NASH may be the result of injury from lipid peroxidation or represent an adaptive change[84]. Glycogenated nuclei are vacuolated nuclei usually observed in periportal hepatocytes. Their presence in biopsies with steatohepatitis is supportive of nonalcoholic etiology (obesity and/or diabetes) because they are very rarely seen in biopsies of alcoholic steatohepatitis (ASH)[85].

Finally, hepatic siderosis might be seen in NAFLD biopsies. It is usually mild (1+, 2+) and occurs in periportal hepatocytes and/or pan-acinar reticulo-endothelial cells. The significance of liver tissue iron deposition and abnormal iron metabolism genetics in the pathogenesis and progression of NAFLD are under investigation[86,87].

HISTOLOGY OF NAFLD IN ADULTS: PATHOLOGIST REPRODUCIBILITY

Reproducibility studies have shown excellent to good agreement for the extent of steatosis, presence of perisinusoidal fibrosis, and stage of fibrosis in adult NAFLD; the feature with the least agreement between histopathologists is lobular inflammation[54,88,89]. Intra-observer agreement is generally better than inter-observer agreement for all histological features and diagnostic categories.

HISTOLOGICAL FINDINGS OF NAFLD/NASH IN CONCURRENCE WITH OTHER LIVER DISEASE

The increased frequency of NAFLD in the general population makes it important for pathologists to recognize the lesions, especially those of NASH, when concurrent with other clinically suspected liver diseases. The frequency of co-existent NASH in a series of > 3000 non-allograft liver biopsies undertaken for clinical suspicion of CHC, autoimmune hepatitis, hemochromatosis, alpha-1-antitrypsin deficiency, or chronic hepatitis B was 2.6%[90]. This number most likely underestimates the actual prevalence of the concurrence given the very stringent criteria for the diagnosis of SH used in the study[56]. Other groups, however, have found similar results[91].

In CHC, which is the most common chronic liver disease diagnosed with concomitant NASH (5%-10%)[90-93], the presence of NAFLD can accelerate disease progression and decrease response to antiviral treatment with negative consequences on patients' prognoses[94,95]. Other studies have shown that co-existent fatty liver or NASH in CHC, more common in patients with genotype 3, was not related to metabolic disturbances[93]. In a subsequent study, however, this same group showed that insulin resistance was increased in CHC, particularly genotypes 1 and 4, and was independently related to advanced fibrosis[94]. Thus, the data for CHC and NAFLD is still under investigation.

The diagnosis of concurrent NASH can be a challenge. One study required the identification of the characteristic zone 3 perisinusoidal fibrosis[90], as this is not a typical finding in other forms of chronic liver disease; another study only required the prominence of steatosis and inflammation in zone 3, along with hepatocellular ballooning[93]. It is important to understand that hepatocellular ballooning, steatosis, and even MDB, have been documented in CHC[96].

Recently, the co-existence of NAFLD and alcoholic liver disease (ALD) has been increasingly recognised in clinical settings. The presence of NAFLD in alcoholic patients has been linked with progression of ALD[97], while some have shown that moderate alcohol consumption is associated with fibrosis progression in NAFLD[98]. Interestingly, in contrast, several studies have shown a beneficial effect of moderate alcohol intake. Moderate alcohol use was shown to be protective against insulin resistance in morbidly obese individuals[73], was associated with decreased prevalence of fatty liver in asymptomatic individuals[99], and with decreased prevalence of steatohepatitis in NAFLD patients[100]. The pathologist is not able to recognize the relative contributions of NAFLD or ALD to the liver injury in a biopsy with steatohepatitis.

HISTOLOGICAL FINDINGS OF RESOLUTION OF NASH

Resolution of the histological lesions of NASH is one of the primary end-points of many treatment trials. It is now apparent that "spontaneous" resolution of liver injury can occur without treatment (i.e. studies of placebo groups)[101-103]. This observation, therefore, indicates the need for adequate sample size as well as randomized controlled trial for treatment trials of NASH.

Improvement of the major histological features of disease activity (i.e. steatosis, lobular inflammation, and ballooning), grade of steatohepatitis and, occasionally, of fibrosis following therapy using different agents, including thiazolidinediones (pioglitazone and rosiglitazone), metformin, vitamins E and C, dietary interventions, or bariatric surgery have been reported[104]. However, after discontinuation of medical treatment, the initial histological improvement might not be maintained, indicating the need for longer (permanent?) duration of therapy[105]. Nevertheless, the results of one long-term treatment trial with the insulin sensitizer roziglitazone showed that there is no additional anti-steatogenic effect with longer treatment, despite an increase in insulin sensitivity[106]. In contrast, reportedly, surgical intervention for obesity appears to have long-lasting beneficial effects on liver histology[107,108].

Histological evaluation of post-treatment liver biopsies in a small trial[102] showed that increased portal inflammation is a feature related to resolution of NASH; a subsequent review of published treatment trials has confirmed this finding, which is independent of the type of therapeutic intervention (medical, dietary, or surgical)[56]. In some cases, successful treatment with resolution is associated with the change in the quality of zone 3 perisinusoidal fibrosis from dense to delicate[102].

HISTOLOGY OF NAFLD IN PEDIATRIC SUBJECTS

Diagnosis of nonalcoholic fatty liver disease in children continues to be a challenge for pathologists. Clinicopathological studies have confirmed that the lesions may or may not resemble those of adults, including cirrhosis with and without steatosis and inflammation[109-119].

Utilizing a mathematic algorithm of the lesions following evaluation of 100 obese children and their liver biopsies, Schwimmer *et al*[120] proposed the currently utilized schema of types 1 and 2 steatohepatitis in pediatric fatty liver disease. Type 1, the least common overall, but the most common in girls, resembles the adult pattern, with zone 3 accentuation of steatosis. Type 2 is more common in boys, and consists of either zone 1 accentuation of steatosis, or panacinar steatosis. Ballooning was uncommon in both types. Zone 3 perisinusoidal fibrosis was uncommon in type 2, whereas, when fibrosis was present, only portal-based fibrosis was seen. A small percent of cases did not fit completely into either category in the Schwimmer study, and were referred to as "overlap". A subsequent clinicopathological series from children from a single site in Rome[112], as well as multiple centers across North America[115], have found types 1 and 2 less common than overlap patterns, and individual features such as zone 3 steatosis, ballooning, and zone 3 perisinusoidal fibrosis less common in children than in adults[121]. However, no author has yet specified the features and/or patterns of injury that would result in an unequivocal diagnosis of "steatohepatitis" in pediatric cases if there were changes other than the adult-pattern. Thus, this is an area of ongoing investigation in pathology. In addition, there are no natural history studies to date showing evolution of the pediatric pattern in children; whether it evolves to the adult pattern with increasing age or not is unknown. The rate and processes involved in progression to cirrhosis, an unfortunate but growing problem[114] in obese children, are also unknown.

HISTOLOGY OF NAFLD IN SPECIAL POPULATIONS

Asians

It is recognized that the body habitus of individuals of Asian descent differs from that of Caucasoids; there is greater body fat for a given body mass index (BMI) in Asians[122], thus, the prevalence and incidence of NAFLD and advanced fibrosis are related to factors other than BMI. However, histological features, as described, do not appear to differ from those described for Caucasian adults.

Bariatric patients

To date, there is no dedicated literature related to the bariatric population, other than straightforward observations of changes of steatosis, inflammation, and fibrosis following surgery. Many studies are done without a specific protocol requiring follow-up biopsies, and most workers do not require pre and post biopsies to be obtained from the same lobe of the liver, or for surgical biopsies to be obtained as early as possible in the operation to avoid the accrual of polymorphonuclear leukocytes within the lobules (i.e. surgical hepatitis). The former is important because of potential differences between the lobes (capsule: parenchyma ratio) and the latter for obtaining correct lobular inflammation scores. The extant literature is reviewed[108] and highlights the fact that the majority of bariatric patients have varying amounts of steatosis. In addition, a recent study of surgeon ability to detect steatosis and/or cirrhosis has shown that this is not reproducible; thus reinforcing the need for liver biopsy in all bariatric patients[123]. To this, the authors would add unpublished personal observations of the non-cirrhotic biopsies from bariatric surgery, that (1) it is common to see enlarged, prominent portal tracts from biopsies obtained from the left lobe; (2) steatosis is commonly mild and often not in zone 3; and (3) zone 3 perisinusoidal fibrosis is uncommon. It is possible that the very low calorie diet that commonly precedes the actual surgical procedure influences

NAFLD and even NASH that may have been present. Finally, a large European study has shown that after five years, if insulin resistance has not improved, fibrosis might actually show progression[124].

NASH IN THE TRANSPLANTED LIVER

NAFLD can occur in the allograft liver as recurrence or as a *de novo* process[125-136].

The incidence of recurrent steatosis in patients transplanted for cryptogenic cirrhosis or NASH-cirrhosis ranges from 25%-100%, while NASH development is observed in 10%-37.5% of these cases without leading to early allograft failure (reviewed in[135]). Recurrent NASH can progress to advanced fibrosis and cirrhosis, but only rare cases have been re-transplanted as a result of decompensation due to severe NASH in the allograft[137].

Development of *de novo* steatosis and/or NASH in the liver allograft might be the result of metabolic disturbances, as transplanted patients are at increased risk for obesity, diabetes, hypertension, and hypercholesterolemia and have decreased physical activity levels. Moreover, anti-rejection medications, in particular steroid treatment and cyclosporine, are known risks for fatty liver development in the allograft, probably by affecting diabetogenic pathways[51,138]. Therefore, the occurrence of NASH in an allograft liver of a patient transplanted for "cryptogenic cirrhosis" does not justify attribution of the original disease to "burned-out" NASH without appropriate clinico-pathological correlation[139-141].

DIFFERENTIAL DIAGNOSIS

Clinico-pathological correlation is essential for highlighting the underlying cause of steatohepatitis in a liver biopsy, as the histology is similar irrespective of etiology. The differential diagnoses of NAFLD and NASH include ASH, drug toxicity (tamoxifen, glucocorticoids, and highly active antiretroviral therapy in human immunodeficiency virus patients), metabolic diseases (such as Wilson disease, tyrosinemia, and citrin deficiency), lipodystrophy, surgical procedures (such as jejunoileal bypass and biliopancreatic diversion), total parenteral nutrition, and malnutrition[51].

ASH vs NASH

The ongoing discussions of the limits of alcohol intake in "nonalcoholic" fatty liver[142] revolve around the complex interactions of host (genetic and metabolic) factors[143,144], the type of alcohol utilized, patterns of drinking[144], and the potentially beneficial interactions of modest alcohol with insulin resistance[73].

Hepatopathologists agree that the histology of uncomplicated NAFLD and alcoholic steatosis are indistinguishable. Further, NASH might be indistinguishable from ASH; however, there are some histological features observed in ALD that have not yet been described in NAFLD. These include sclerosing hyaline necrosis (a combination of dense perivenular fibrosis, MDB accumulation, and hepatocyte necrosis in zone 3), veno-occlusive lesions, alcoholic foamy degeneration (zone 3 microvesicular steatosis), and cholestasis[51]. Generally, there is consensus that the overall histopathological appearance of NASH is milder than that observed in ASH[145], with the understanding that alcoholic steatosis can be as "mild" as NAFLD.

Certain histological lesions, including severe steatosis, glycogenated nuclei, and lipogranulomas, are more frequent in NAFLD compared to ALD; however, their utility in differential diagnosis on an individual basis is limited[145]. On the other hand, numerous, well formed MDB and dense fibrosis, composed mostly of collagen type Ⅲ might be indicative of an alcoholic rather than metabolic origin[146-148]. One group utilized immunohistochemical detection of the insulin receptor (IR) and increased expression of protein tyrosine phosphatase 1B (PTP1B), a protein that acts as a negative regulator of IR expression, to aid in the histological distinction of ASH and NASH. IR receptor was more frequent in NASH in contrast to normal expression for IR in conjunction with low level expression of PTP1B noted in ASH[149]. The clinical utility of this method, however, has not been tested. The same group also proposed a noninvasive model for the differential diagnosis between ALD and NAFLD using the ALD/NAFLD Index (ANI). This is based on the mean corpuscular volume, aspartate aminotransferase (AST)/ALT ratio, BMI and gender. An ANI greater than 0 indicates ALD, while ANI less than 0 is diagnostic of NAFLD[150]. There has been no published validation of this test.

GRADING AND STAGING OF THE HISTOLOGICAL LESIONS IN NAFLD

The first system for grading necroinflammatory activity and staging fibrosis in NASH appeared in 1999[53] in response to the growing need for standardization of the histological criteria used for the diagnosis of NASH and its differentiation from steatosis, and to meet the necessity for the development of a histological scoring system similar to those already used for other forms of chronic hepatitis. In this scoring system, the features that were shown to contribute the most to the severity of the disease, such as steatosis, lobular and portal inflammation, and hepatocellular ballooning were semi-quantitatively assessed to produce a three-tier global grade of activity (Table 1). Staging was based on the characteristic pattern and evolution of fibrosis in NASH with initial involvement of perisinusoidal spaces in zone 3 (stage 1) and subsequent development of portal/periportal fibrosis (stage 2), bridging fibrosis (stage 3), and cirrhosis (stage 4) (Table 1)[53].

Among subsequently published scoring systems for grading histological activity in NAFLD[54,151] and NASH[152], the current most widely used histological scoring system is the one published in 2005 by the NASH Clinical Research Network (CRN), sponsored by the National Institute of Diabetes, Digestive and Kidney Diseases[54]. The NASH CRN system has several advantages: it is a validated system created from blinded reviews by nine pathologists, it is applicable to the entire histological

Table 1 Grading activity and staging fibrosis in nonalcoholic steatohepatitis according to Brunt *et al* (adapted from[53,54])

	Steatosis (1: ⩽ 33%, 2: 33%-66%, 3: ⩾ 66%)	Ballooning (zonal location and severity recorded)	Inflammation[a]	
			L-Lobular [(0-3): 0: absent, 1: < 2, 2: 2-4, 3: > 4 foci]	P-Portal [(0-3): 0: absent, 1: mild, 2: moderate, 3: severe]
Grade 1 (mild)	1-2	Minimal, zone 3	L = 1-2	P = 0-1
Grade 2 (moderate)	2-3	Present, zone 3	L = 1-2	P = 1-2
Grade 3 (severe)	2-3	Marked, predominantly zone 3	L = 3	P = 1-2

[a]Counted in 20 × fields. Stage of fibrosis: 1: Zone 3 perisinusoidal fibrosis, focal or extensive; 2: As above with focal or extensive periportal fibrosis; 3: Bridging fibrosis; 4: Cirrhosis, probable or definite.

Table 2 Nonalcoholic steatohepatitis Clinical Research Network system for scoring activity and fibrosis in nonalcoholic fatty liver disease (modified from[54])

Steatosis grade (S)	Lobular inflammation[a] (L)	Hepatocyte ballooning (B)
0: < 5%	0: None	0: None
1: 5%-33%	1: < 2	1: Few ballooned cells
2: 34%-66%	2: 2-4	2: Many ballooned cells
3: > 66%	3: > 4	

[a]Counted in 20 × fields. Nonalcoholic fatty liver disease Activity Score: S + L + B (range 0-8). Stage of fibrosis (Fibrosis score): 0: None; 1: 1a, mild, zone 3 perisinusoidal fibrosis, 1b, moderate, zone 3 perisinusoidal fibrosis, 1c, portal/periportal fibrosis only; 2: Zone 3 perisinusoidal fibrosis and portal/periportal fibrosis; 3: Bridging fibrosis; 4: Cirrhosis, probable or definite.

spectrum of NAFLD, it scores both adult and pediatric NAFLD biopsies, and it generates a numeric score for grading activity and NAFLD activity score (NAS) for comparing pre- and post-treatment biopsies in therapeutic trials. The NAS derives from the summation of individual scores for steatosis, lobular inflammation, and hepatocellular ballooning and ranges from 0 to 8 (Table 2). In the validation study, NAS of 1 or 2 corresponded to definitely not NASH, while a NAS score 5-8 correlated with definite NASH. Activity scores 3 and 4 were noted in borderline cases that did not fulfill the pathologists' criteria for definite NASH. It is important to note, however, that the numbers generated by NAS were not created to be used as a substitute for histological diagnosis; properly utilized, the NAS is applied by the pathologist after reaching final diagnosis.

Fibrosis scoring with the NASH CRN system is based on the prototype staging method proposed by Brunt *et al*[53], with the difference that stage 1 is further subdivided into three sub-stages to differentiate between delicate perisinusoidal zone 3 fibrosis (stage 1a), dense perisinusoidal zone 3 fibrosis (stage 1b), and portal fibrosis only (stage 1c)[54]. NASH CRN stage 1c refers to the pattern of fibrosis sometimes seen in severely obese patients and in pediatric NASH (Table 2). The value of histological scoring systems for grading and staging in NAFLD in routine practice has not yet been adequately assessed and, to date, they are mainly used in treatment trials and in natural history studies. Furthermore, the recognition of the significance of other histological features associated with definite NASH and advanced fibrosis, such as portal chronic inflammation[70], and the proposed use of serum biomarkers of hepatocyte apoptosis for risk-stratifying patients with NAFLD[41], indicate that the currently used systems could be improved by including these histological features in the semi-quantitative histological scoring of both adult and pediatric NAFLD[58].

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ORIGINAL ARTICLE

Autologous CD34$^+$ and CD133$^+$ stem cells transplantation in patients with end stage liver disease

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Abstract

AIM: To assess the utility of an autologous CD34$^+$ and CD133$^+$ stem cells infusion as a possible therapeutic modality in patients with end-stage liver diseases.

METHODS: One hundred and forty patients with end-stage liver diseases were randomized into two groups. Group 1, comprising 90 patients, received granulocyte colony stimulating factor for five days followed by autologous CD34$^+$ and CD133$^+$ stem cell infusion in the portal vein. Group 2, comprising 50 patients, received regular liver treatment only and served as a control group.

RESULTS: Near normalization of liver enzymes and improvement in synthetic function were observed in 54.5% of the group 1 patients; 13.6% of the patients showed stable states in the infused group. None of the patients in the control group showed improvement. No adverse effects were noted.

CONCLUSION: Our data showed that a CD34$^+$ and CD133$^+$ stem cells infusion can be used as supportive treatment for end-stage liver disease with satisfactory tolerability.

Key words: CD34; CD133; Stem cell; Liver; Hepatitis C virus

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INTRODUCTION

Cirrhosis is the end stage of chronic liver injury caused by viral hepatitis or alcohol intake. In Egypt, most malignant neoplasms of the liver arise on top of chronically damaged livers, typically a cirrhotic liver due to hepatitis C

virus (HCV) infection. Two main factors are responsible for the irreversibility of cirrhosis. One factor involves the increased and continuous deposition of the extracellular matrix, due to increased collagen synthesis accompanied by insufficient breakdown of collagen. The other is the impaired capability of the liver to regenerate, which predisposes a patient to postoperative dysfunction or liver failure. Previous studies have shown that a fibrotic liver following hepatic resection regenerates very slowly in comparison with a normal liver. However, little is known about the mechanism(s) of retarded liver growth; though, it has been postulated that growth factors can promote cirrhotic regeneration by an as yet unidentified mechanism.

Until now, effective treatments capable of reversing cirrhosis have not been developed. In the last few years, there has been increasing evidence that adult cells have far greater differentiation plasticity than previously thought, with bone marrow turning into skeletal muscle[1] and skeletal muscle back into bone marrow (BM)[2]; brain into blood[3] and back again[4,5]; BM into liver[6,7] and back again[8]. These phenomena occurred in different mammals (including mice, rats, and sheep); however, it is difficult to know the human relevance of the findings without studying human tissues.

In two recently published studies[9,10], the effects of CD34 hematopoietic stem cell intrahepatic injection and whole BM peripheral infusion have been evaluated in five and nine cirrhotic patients; respectively. The results of these two studies showed that BM stem cells transplantation improved the residual liver function in cirrhotic patients. In the study of Gordon *et al*[9], a decrease in serum bilirubin and an improvement in serum albumin were observed in three and four of the five patients, respectively, with disappearance of ascites in one patient only.

Similarly, Terai *et al*[10] reported significant increases in albumin and total protein levels, and an improvement of the Child-Pugh score in all of the nine patients, and a reduction in ascites in six patients. These results, which originated from ongoing research on BM stem cells and the liver, may be considered promising and encouraging, as they demonstrate that clinical results might sometimes precede scientific evidence. However, a more cautious analysis of the results is necessary, considering the clinical course and potential new therapies for complex disorders such as human liver cirrhosis.

These two studies[9,10], however, were conducted on small groups of patients, were not randomized, and also lacked a control group of stem cell untreated patients. The presence of a control group, in particular, is of utmost importance in clinical studies investigating the efficacy of potential new therapies. Using a control group (that should be homogeneous to the experimental group for both epidemiological and clinical characteristics) reduces the likelihood that modifications observed during treatment are accidental and not directly dependent on the therapy administered, and randomization increases the probability that other variables, not considered in the study design, are distributed evenly in both the experimental and control groups.

In the current study, we investigated the possibility of using $CD34^+$ and $CD133^+$ stem cells as a therapeutic modality in patients with end-stage liver diseases.

MATERIALS AND METHODS

Ethics

The ethical committees of Kaser El-Aini School of Medicine and the National Cancer Institute, Cairo University, approved the study protocol and a written informed consent was obtained from each patient.

Studied groups

The present study includes 140 patients with end-stage liver cirrhosis who attended the Hepatology Clinic in Kaser El-Aini School of Medicine, Cairo University, from June 2008 to May 2009.

Patients were randomized into one of two groups: Group 1: 90 patients who received granulocyte colony stimulating factor (G-CSF) (Neupogen, Roche) for five days, followed by autologous $CD34^+$ and $CD133^+$ stem cell infusion in the portal vein; Group 2: 50 patients who served as a control and those received regular liver treatment.

Inclusion criteria

Patients were included in this study if they fulfilled the following criteria: male or female patients aged from 20 to 60 years with evidence of chronic liver insufficiency (decreased serum albumin and/or increased bilirubin and/or increased international normalized ratio-INR), patients unlikely to receive a liver transplant and who had a World Health Organization (WHO) performance score of less than 2 with an ability to give a written consent.

Exclusion criteria

Patients were excluded if they were below the age of 20 or above the age of 60 years, pregnant or lactating women, those with recent recurrent gastrointestinal bleeding, hepatocellular carcinoma or spontaneous bacterial peritonitis, an evidence of human immunodeficiency virus or other life threatening infection, unable to give a written consent, having history of hypersensitivity to G-CSF, or included in any other clinical trial within the previous month. Patients were randomized into either one of these two groups.

Patients in both groups were comparable for baseline characteristics: age and sex, etiology of liver disease and model for end-stage liver disease (MELD) score.

Clinical evaluation was done for all patients in the study, including a detailed medical history and complete clinical examination, with special emphasis on the presence markers of liver cell failure e.g. ascites, jaundice, LL.edema, bleeding tendency, and signs of encephalopathy in addition to the WHO performance score. Abdominal ultrasound scanning was done for both groups using a Hitachi 515 real time scan after overnight fasting.

Laboratory investigations were done for all patients, and included liver biochemical profile [serum bilirubin

and albumin, prothrombin time and concentration, INR, alanine transaminase (ALT) and aspartate transaminase (AST)]; blood urea and serum creatinine levels; blood sugar (fasting and two hours post-prandial); complete blood analysis and α-fetoprotein, as well as a hepatitis serological profile (HBsAg, HBcAb, HCV Ab, HCV RNA by quantitative polymerase chain reaction). The MELD score was calculated to assess the degree of hepatic decompensation for every patient.

Treatment regimen

Group 1: From days one to five, patients received a daily subcutaneous injection of granulocyte colony stimulating factor (G-CSF at 300 μg Neupogen (Roche) - 300 μg/vials) for five days to increase the number of circulating hematopoietic cells. Blood samples were then taken for hematology, biochemical analysis, and coagulation profile on days one and five. Data regarding symptoms and adverse events were collected and recorded. Patients were admitted to the hospital and underwent BM aspiration on day six.

BM stem cell aspiration

Patient's skin was cleaned with 70% alcohol at the iliac crest, which is the usual site for puncture in adults. Skin, subcutaneous tissues, and periosteum overlying the selected site for puncture were infiltrated with xylocaine local anesthesia and serial punctures from multiple sites were performed.

With a boring movement, needles (Salah and Klima) were passed perpendicularly into the cavity of the ileum at a point just posterior to anterior superior iliac spine or 2 cm posterior and 2 cm inferior to the anterior superior iliac spine to aspirate 250 mL of the BM.

Following collection, the BM products were transferred to the stem cell laboratory for immuno-magnetic purification of the $CD34^+$ and $CD133^+$ stem cell population. $CD34^+$ and $CD133^+$ cells were isolated using the positive cell selection kit (MACS System Milteny Biotec, Gmbh, Germany (http://www.miltenyibiotec.com). Collected cells were washed twice with phosphate buffered saline pH 7.4, supplemented with 0.5% bovine serum albumin and 5 mmol/L EDTA, and centrifuged at 1500 r/min for 10 min at 4℃. The cell suspension was counted, adjusted to 0.5×10^8, centrifuged, and re-suspended in 100 mL physiological saline.

Patients were admitted to hospital and infused, while fasting, with the purified human stem cells into the portal vein (as previously determined by duplex Doppler scan to assess its potency) under ultrasound guidance using, diazepam 10 mg IV for sedation and xylocaine local infiltration anesthesia.

Group 2: A daily SC injection of distilled water was given for all patients while receiving their usual supportive measures before and after injection.

Post-treatment follow up of the patients

All patients in both groups were followed up every hour for 24 h, then every week for the first month, and regularly every month for 6 mo. During the follow up, patients were observed for the following: serum bilirubin and albumin, prothrombin time and concentration, INR, ALT and AST; blood urea and serum creatinine levels; blood sugar (fasting and two hours post-prandial); complete blood analysis, together with estimation of the degree of ascites, Child-Pugh score, performance score, hepatic encephalopathy and hematemesis.

Statistical analysis

All patients' data were tabulated using Excel XP. Data were processed by SPSS (Statistical Package for Science and Society) version 12.0 for Windows XP. The descriptive statistics were presented as mean ± SD for quantitative variables. All qualitative data were expressed by frequency (number) and percent. Comparisons between groups were done using χ^2 test, Fischer's Exact test or McNemar test when appropriate for qualitative data but independent sample *t* test and paired sample *t* test were used for normally distributed quantitative variables, while the non-parametric Mann Whitney test and Wilcoxon singed ranks test were used for abnormally distributed quantitative variables. Percent changes in prognostic variables (e.g. albumin) were calculated and compared using the Mann Whitney test. In all tests, *P* values lower than 0.05 were considered as statistically significant. Also, linear regression analysis was employed to determine the degree of improvement of clinical features in relation to response rate to the treatment. Multivariate analysis also showed no significant correlation between HCV viral titer changes and serum albumin, prothrombin concentration (PC), Child score, Performance score ascites grade, and hematemesis attack.

RESULTS

One hundred and forty patients (117 males and 23 females) with a mean age of 50.27 ± 6.05 years (range; 45 to 59 years) were included in the study; 90 of them received autologous, BM-derived $CD34^+$ and $CD133^+$ cell transplantation, and 50 were included as a control. In all studied patients, the cells were injected into the portal vein under ultrasound guidance. The etiology, diagnosis, clinical signs, results of investigations, cell concentrations, and post infusion observations for each patient are shown in Tables 1 and 2. After G-CSF treatment, increased total white blood cell counts indicated mobilization of progenitor cells into the circulation in all patients. Patients were then given 0.5×10^8 $CD34^+$ and $CD133^+$ cells as a single bolus.

No major complications, either local or systemic, occurred as a result of the stem cell transplantation, except for mild pain and discomfort at the site of $CD34^+$ and $CD133^+$ cell infusion. However, a short term fever developed in 15 patients, which subsided in 24 h. Mild bone pain developed in 23 patients after receiving G-CSF, which also subsided spontaneously. However, there was no bleeding, infection, or significant deterioration in liver

Table 1 Clinical features of the studied groups *n* (%)

Variables	Treated group (*n* = 90)	Control group (*n* = 50)
Age (yr, mean ± SD)	50.27 ± 6.05	50.9 ± 7.23
Sex		
Male	78 (86.7)	39 (78)
Female	12 (13.3)	11 (22)
Residence		
Urban	30 (33.33)	20 (40)
Rural	60 (66.66)	30 (60)
Jaundice	36 (40)	21 (42)
Encephalopathy	21 (23.33)	15 (30)
Weight loss	57 (63.33)	29 (58)
Ascites and lower limb edema	78 (86.66)	41 (82)
Peripheral erythema	75 (83.33)	39 (78)
Bleeding tendency	48 (53.33)	26 (52)
Hematemesis	24 (26.66)	12 (24)
Quantitative HCV RNA PCR (IU/mL)	1 128 230-1 810 530	1 235 650-1 754 150

HCV: Hepatitis C virus; PCR: Polymerase chain reaction.

Table 2 Ultrasonographic features of the studied groups *n* (%)

Variables	Treated group (*n* = 90)	Control group (*n* = 50)
Liver size		
Average	27 (30)	16 (32)
Shrunken	54 (60)	23 (46)
Enlarged	9 (10)	11 (22)
Liver texture		
Cirrhotic	90 (100)	50 (100)
Focal lesions	0	0
Spleen		
Average sized	24 (26.66)	12 (24)
Mild splenomegaly	48 (53.33)	29 (58)
Moderate splenomegaly	18 (20)	9 (18)
Huge splenomegaly	0	0
Splenectomy	0	0
Ascites		
Absent	6 (6.7)	8 (16)
Mild	12 (13.3)	5 (10)
Moderate	63 (70)	32 (64)
Massive	9 (10)	5 (10)

Table 3 Biochemical changes in the studied groups (mean ± SD)

	Pre treatment	1st month	2nd month	3rd month	6th month
Bilirubin (mg%)					
Patients	2.51 ± 1.94	2.75 ± 1.66	2.31 ± 1.33	2.34 ± 1.29	2.18 ± 1.28
Control	2.68 ± 1.49	2.80 ± 1.51	2.92 ± 1.71	3.41 ± 1.77	3.58 ± 1.56
			$P < 0.001$	$P < 0.002$	$P < 0.003$
Albumin (gm/dL)					
Patients	2.61 ± 0.44	2.73 ± 0.62	2.83 ± 0.39	2.92 ± 0.33	2.94 ± 0.45
Control	2.69 ± 0.26	2.60 ± 0.30	2.54 ± 0.25	2.44 ± 0.35	2.10 ± 0.34
				$P < 0.04$	$P < 0.001$
PC (%)					
Patients	46.2 ± 12.5	49.6 ± 12.5	55.0 ± 14.5	54.9 ± 12.9	56.1 ± 15.7
Control	45.1 ± 12.8	44.9 ± 13.1	42.2 ± 13.5	40.9 ± 14.1	35.8 ± 15.9
		$P < 0.005$	$P < 0.005$	$P < 0.005$	$P < 0.005$
AST (U/L)					
Patients	78.2 ± 32.1	67.4 ± 25.2	68.0 ± 20.2	61.0 ± 18.7	69.0 ± 29.2
Control	75.4 ± 35.2	77.2 ± 30.1	72.1 ± 32.3	75.5 ± 33.3	80.2 ± 32.4
		$P < 0.002$	$P < 0.002$	$P < 0.003$	$P < 0.003$
ALT (U/L)					
Patients	51.5 ± 25.5	45.7 ± 19.6	50.9 ± 27.7	42.8 ± 16.1	47.0 ± 21.3
Control	60.1 ± 20.4	62.5 ± 21.2	66.1 ± 25.1	63.5 ± 23.7	23.7 ± 22.1
		$P < 0.001$	$P < 0.001$	$P < 0.01$	$P < 0.01$

PC: Prothrombin concentration; AST: Aspartate transaminase; ALT: Alanine transaminase.

function. CT scans showed no evidence of focal liver lesions in any of the treated patients, either before or after infusion. Duplex Doppler ultrasound scans showed patent portal veins with normal hepato-pedal flow.

Monthly assessment of changes in the serum level of bilirubin, albumin, liver enzymes, and fluid collection was performed. Serum albumin, bilirubin, transaminases, prothrombin time and concentration, together with estimation of the degree of ascites, Child-Pugh score, performance score, hepatic encephalopathy, and hematemesis were performed every week for the first month, then every month up to 6 mo (Table 3).

Near normalization of liver enzymes and improvement in the synthetic function of the liver were observed in 54.5% of $CD34^+$ and $CD133^+$ transfused patients and 13.6% of them showed stable states. In comparison, none of the control group showed any improvement (P = 0.069 and 0.023, Table 4 and Figure 1A-E).

None of the responders or patients with stable disease showed progressive or increased ascites or needed taping, and the treatment schedule for diuretic therapy was dramatically reduced. Disappearance of ascites, as demonstrated by ultrasound scan, was detected in 21 patients, and improvement in the degree of ascites was reported in 51 patients (Figure 2A and B). Also, an improvement in Child-Pugh score was detected in 48 patients (Figure 3A and B); performance score in 52 patients (Figure 4A and B). Improvements in hepatic coma and hematemesis (Table 5) were detected in 91.3% and 87%, respectively. Similarly, a significant improvement

Table 4 Response rate of the treated group according to synthetic and biochemical liver functions *n* (%)

Marker	Responders				Stable				Non-responders				*P* value
	1st mo	2nd mo	3rd mo	6th mo	1st mo	2nd mo	3rd mo	6th mo	1st mo	2nd mo	3rd mo	6th mo	
Bilirubin	24 (26.6)	30 (37.0)	33 (46.5)	39 (56.5)	0 (0)	9 (11.1)	9 (12.8)	0 (0)	66 (73.3)	42 (51.9)	29 (40.8)	30 (43.5)	0.516
Albumin	57 (63.3)	45 (55.6)	42 (59.2)	40 (58.0)	3 (3.33)	9 (11.1)	9 (12.7)	8 (11.6)	30 (33.3)	27 (33.3)	20 (28.1)	21 (30.4)	0.069
PC	66 (73.3)	57 (70.4)	47 (66.2)	48 (65.6)	0 (0)	3 (3.6)	6 (8.5)	0 (0)	24 (26.6)	21 (25.8)	18 (25.4)	21 (30.4)	0.023
AST	57 (63.3)	48 (60.3)	47 (66.2)	39 (56.5)	3 (3.33)	0 (0)	0 (0)	0 (0)	30 (33.3)	33 (40.7)	24 (33.8)	30 (43.5)	0.673
ALT	54 (60.0)	48 (59.2)	47 (66.2)	30 (43.5)	0 (0)	3 (3.7)	6 (8.5)	6 (8.7)	36 (40.0)	30 (37.1)	18 (25.3)	33 (47.8)	0.550

PC: Prothrombin concentration; AST: Aspartate transaminase; ALT: Alanine transaminase.

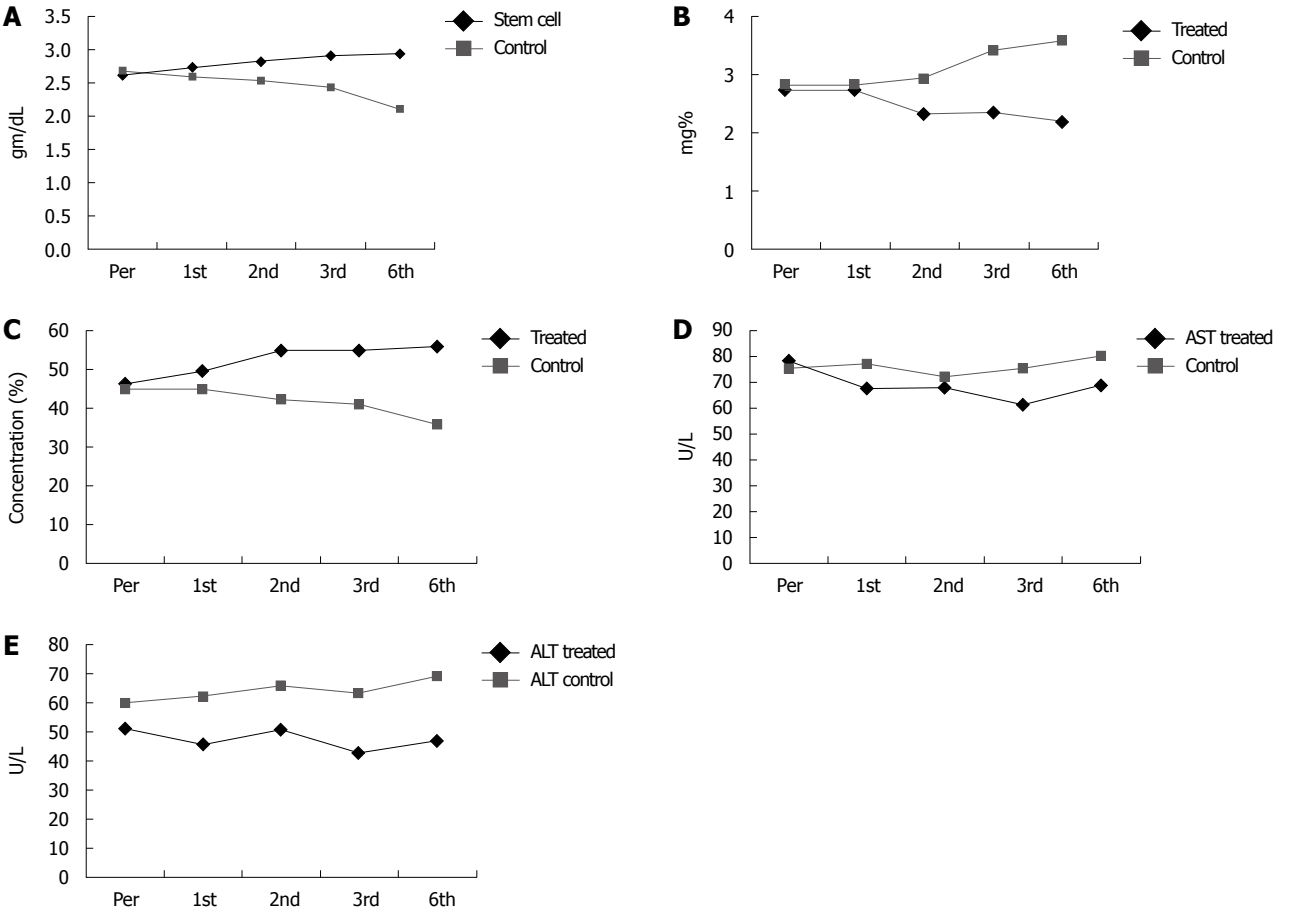

Figure 1 Changes in serum albumin (A), serum bilirubin (B), prothrombin concentration (C), aspartate transaminase values (D) and alanine transaminase values (E) in both control and treated groups. Per: Pre-treatment; 1st: First month; 2nd: Second month; 3rd: Third month; 6th: Sixth month after treatment.

was observed in the group of responders compared to the other two groups (Table 6, Figures 2 and 3).

HCV viral titer

In the study population, the mean baseline HCV viral titer value was 1128.23 ± 1810.53 IU/mL. One month after stem cell therapy, it increased to 2749.70 ± 10533.15 IU/mL, then decreased to 848.88 ± 1375.72 IU/mL two months after the study and then to 592.23 ± 1300.90 IU/mL after three months. After 6 mo of the study, the mean HCV viral titer further decreased to 351.84 ± 1068.73 IU/mL in the treated group. However, no significant change in the viral titer was observed in the control group during the study period.

Linear regression analysis was employed to determine the degree of improvement in the clinical features in relation to the response to treatment. A significant improvement was observed among the responders compared to the control groups (Table 7).

Multivariate analysis showed no significant correlation between HCV viral titer and serum albumin, PC, Child score, performance score, ascites grade or hematemesis. However, there was a significant inverse relation between serum bilirubin levels and HCV titer, which reached the highest significance in the 6th month after treatment ($r = 0.56$, $P < 0.002$).

Survival after stem cell therapy (6 mo follow-up): Nine patients with post-HCV liver cell failure died (seven

Table 5 Cross-table showing progress of hepatic coma and hematemesis after 1, 2, 3 and 6 mo *n* (%)

	Pretreatment (*n* = 90)		1 mo (*n* = 90)		2 mo (*n* = 81)		3 mo (*n* = 71)		6 mo (*n* = 69)	
	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
Coma										
Studied group	60 (66.7)	30 (33.3)	87 (96.7)	3 (3.3)	72 (89)	9 (11)	66 (92.9)	5 (7.1)	63 (91.3)	6 (8.7)
	50 patients		50 patients		44 patients		34 patients		26 patients	
Control group	31 (62)	19 (38)	29 (48)	21 (42)	24 (54.5)	20 (45.5)	14 (41.2)	20 (58.8)	5 (19.2)	21 (80.7)
Hematemesis										
Studied group	72 (80)	18 (20)	90 (100)	0 (0)	75 (92.6)	6 (7.4)	68 (95.8)	3 (4.2)	60 (87)	9 (13)
	50 patients		50 patients		44 patients		34 patients		26 patients	
Control group	35 (70)	15 (30)	32 (64)	18 (36)	24 (54.5)	20 (54.5)	12 (35.3)	24 (70.6)	6 (23)	20 (77)

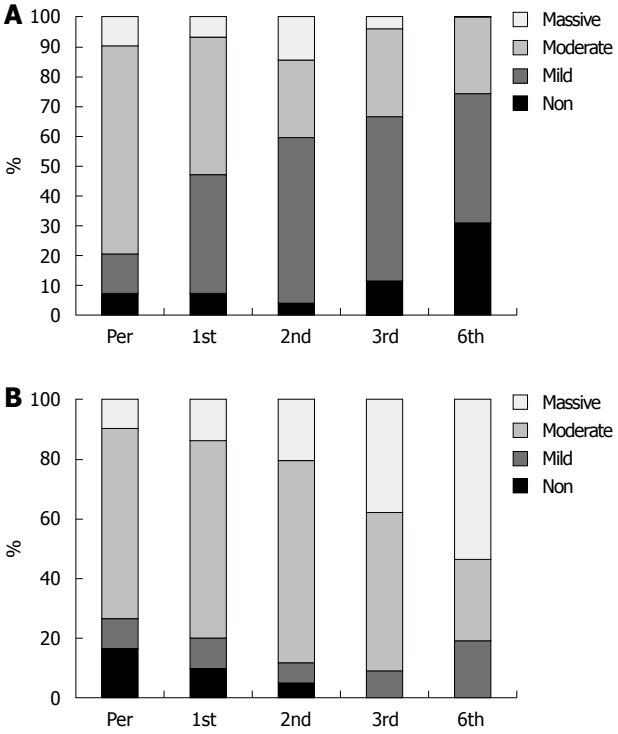

Figure 2 Degree of ascites in the study population (A) and control group (B). Per: Pre-treatment; 1st: First month; 2nd: Second month; 3rd: Third month; 6th: Sixth month after treatment.

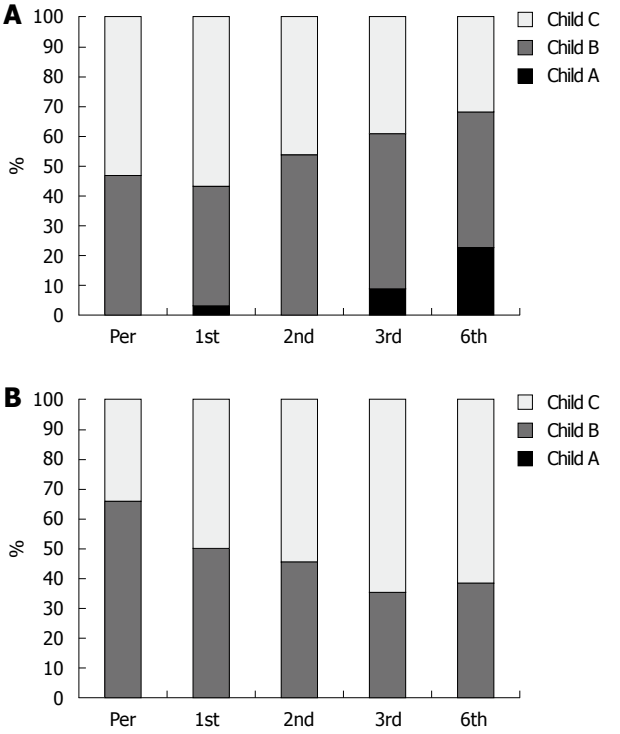

Figure 3 Child-Pugh scores in the study population (A) and control group (B). Per: Pre-treatment; 1st: First month; 2nd: Second month; 3rd: Third month; 6th: Sixth month after treatment.

from attacks of hematemesis, and two from hepato-renal syndrome) and 13 were considered as lost follow up patients, though still alive, because they did not attend at the regular scheduled time for follow-up. In comparison, 26 patients from the control group died (15 from attacks of haematemesis, five from hepato-renal syndrome, and six from hepatic coma) (Figure 5).

DISCUSSION

Until now, liver transplantation has been considered the only curative treatment for decompensated cirrhosis[11]. However, this procedure is limited by technical difficulties, high cost, and also by a lack of donors[12].

Stem cells have recently shown promise in cell therapy because they have the capacity for self-renewal and multilineage differentiation, and are applicable to human diseases. The diseased liver may recruit migratory stem cells, particularly from the bone marrow, to generate hepatocyte-like cells either by transdifferentiation or cell fusion. Transplantation of BMSCs can restore liver mass and function, alleviate fibrosis, and correct inherited liver diseases. Therefore, it can significantly improve the liver function of patients with terminal liver disease, with good safety and effectiveness[13]. BMSCs can be delivered *via* the intraportal vein, systemic infusion, or *via* intraperitoneal, intrahepatic, and intrasplenic routes.

The current study was designed to evaluate the effects of autologous $CD34^+$ and $CD133^+$ hematopoietic stem cell intrahepatic infusion, and to assess the relationship between HCV viral load and the outcome of stem cell therapy, in patients with post HCV liver cirrhosis. This was the basis for conducting a randomized study on 140 patients with chronic HCV liver disease.

Table 6 Response rate of the treated group in relation to clinical features *n* (%)

Marker	Responders				Stable				Non-responders				*P* value
	1st mo	2nd mo	3rd mo	6th mo	1st mo	2nd mo	3rd mo	6th mo	1st mo	2nd mo	3rd mo	6th mo	
Child-Pugh score	9 (10)	18 (22.2)	21 (29.6)	27 (39.1)	72 (80)	48 (59.3)	45 (63.4)	39 (56.2)	3 (10)	15 (18.5)	5 (7.0)	3 (4.4)	0.03
Performance score	18 (20)	27 (33.3)	42 (59.2)	42 (60.9)	51 (56)	36 (44.4)	18 (25.4)	18 (26.1)	21 (23.2)	18 (22.2)	11 (15.4)	9 (13)	0.02
Degree of ascites	33 (36)	42 (51.8)	44 (62.0)	45 (65.2)	48 (53)	24 (29.6)	21 (29.5)	24 (34.8)	9 (10)	15 (18.6)	6 (8.5)	0 (0)	0.02
Hepatitis C coma	27 (30)	24 (29.7)	24 (33.8)	24 (34.9)	63 (70)	54 (66.6)	47 (66.2)	42 (60.8)	0 (0)	3 (3.7)	0 (0)	3 (4.3)	0.001
Haematemesis	18 (20)	21 (25.9)	21 (29.6)	18 (26.1)	72 (80)	57 (70.4)	50 (70.4)	48 (69.6)	0 (0)	3 (3.7)	0 (0)	3 (4.3)	0.03

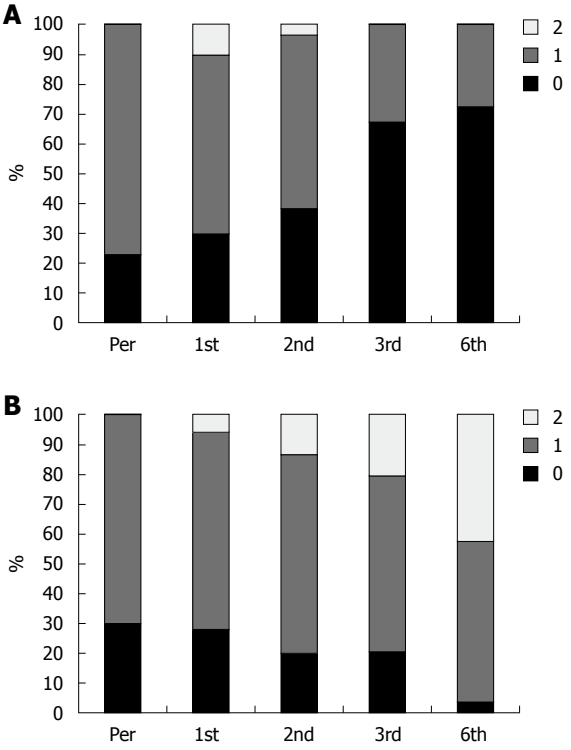

Figure 4 Performance scores in the study population (A) and control group (B). Per: Pre-treatment; 1st: First month; 2nd: Second month; 3rd: Third month; 6th: Sixth month after treatment.

Table 7 Multivariate analysis of hepatitis C virus viral titer *vs* clinical features of the responding group

	Beta	T	*P* value
Child-Pugh score	27.44	1.65	0.03
Performance score	41.2	2.6	0.02
Degree of ascites	35.03	1.3	0.02
Hepatic coma	29.1	1.2	0.001
Haematemesis	26.8	1.4	0.03

Multivariate analysis showing no significant correlation between hepatitis C virus (HCV) viral titer and serum albumin, prothrombin concentration, Child score, performance score, ascites grade, or hematemesis. However, there was a significant inverse relation between serum bilirubin levels and HCV, which reached the highest significance in the 6th month after treatment ($r = 0.56$, $P < 0.002$).

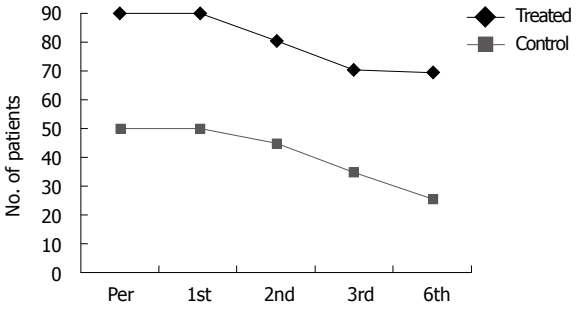

Figure 5 Survival rate in the treated *vs* control group. Per: Pre-treatment; 1st: First month; 2nd: Second month; 3rd: Third month; 6th: Sixth month after treatment.

To improve hepatic homing to the liver, $CD34^+$ and $CD133^+$ progenitor cells were applied into the portal vein directly. Our choice of the portal vein infusion was based on a preclinical model, which reported that HSCs, if applied to the portal vein, demonstrate a high percentage of first-pass entrapment in the liver[14].

We found a significant improvement in the mean serum albumin level in the transplanted patients compared to the control group. Similar results were reported by Gordon *et al*[9]. Similarly, Terai *et al*[10] reported significant improvements in the average serum albumin levels in nine cirrhotic patients that underwent autologous bone marrow cell infusion from the peripheral vein at 24 wk after infusion. Also, Salama *et al*[15] conducted a study on 48 Child C cirrhotic patients and demonstrated that there were borderline significant improvements in the serum albumin levels of the transplanted group compared to the control group at the end of the 6 mo study.

The mean serum bilirubin level showed an improvement in our transplanted patients, starting from the second month after stem cell therapy. These results are also consistent with those of Gordon *et al*[9] and Pai *et al*[13].

In another study, Levicar *et al*[16] isolated and injected 1×10^6 to 2×10^8 $CD34^+$ cells into the portal veins or hepatic arteries of five patients, who were followed up for 18 mo. Four patients showed an initial improvement in serum bilirubin level, which was maintained for up to 6 mo. However, a marginal increase in serum bilirubin was reported in three patients at 12 mo and in one patient, the serum bilirubin increased only at 18 mo post-infusion. They concluded that the beneficial effect seemed to last for around 12 mo.

The mean baseline PC value in our studied population started to improve 1 mo after the procedure, and continued to improve until the 6th month. This improvement was statistically significant. These results agree with those of Salama *et al*[15], who reported that there was an improvement in PC that started in the 2nd month after HSCs transplantation and reached its maximum after 6 mo.

There was a gradual improvement in the hepatic functional reserve of our transplanted patients compared to controls, as assessed by the Child-Pugh score. The maximum improvement in the Child-Pugh score occurred after 6 mo. A statistically significant improvement of Child-Pugh score was demonstrated at four and twenty-four weeks after autologous bone marrow stem cell transplantation therapy by Terai *et al*[10]. In addition, the Child-Pugh score improved significantly in seven out of nine patients studied by Pai *et al*[13].

The quality of life (QOL) in the transplanted patients was assessed using the WHO performance score (PS). There was an improvement in the transplanted patients, as regards PS, starting from the 1st month after the HSCs transplantation until the 6th month post-transplantation.

This improvement has been previously reported by Gordon *et al*[9], who reported a statistically significant improvement in QOL of their patients, as measured by an SF-36 questionnaire after HSCs transplantation.

On the other hand, the present study showed that the number of patients without ascites increased from 6.7% at the start of the study to 27.3% at the end of the study. Maximum improvement occurred after 6 mo, and was of highly significant value as 42 patients (63.6%) showed an improvement in the degree of ascites and 24 patients (36.3%) showed no change in the degree of ascites. However, none of the patients showed deterioration in the degree of ascites. Our results are comparable to those of Terai *et al*[10] and Pai *et al*[13].

At the start of the study, the percent of patients with no history of hepatic encephalopathy was 66.7 % and the percent of patients with history of encephalopathy was 33.3 %. The percent of patients with no attacks of encephalopathy became 96.7 %, 80%, 88.5 % and 91.3% after 1, 2, 3, and 6 mo respectively (maximum improvement occurred after 6 mo). Salama *et al*[15] demonstrated a highly significant improvement in the transplanted group as regards the recurrence of hepatic encephalopathy.

In the study population, the mean baseline HCV viral titer value was 1128.23 ± 1810.53 IU/mL and there was an increase in the transplanted patients during the 1st month only. Thereafter, there was a gradual decrease during the follow-up period. The maximum decrease in HCV viral titer occurred after 6 mo of stem cell transplantation, where 39 patients (59.1%) showed a decrease, 18 patients (27.3%) showed an increase and 9 patients (13.6%) showed no change in their HCV viral titer.

In the study population, the lower pre-transplantation viral load was not due to the intrinsic characteristics of the infective viral strains, but rather to the severity of liver disease. Thus, one month after stem cell transplantation, higher levels of viral replication were restored due to regeneration of liver after stem cell transplantation[17]. Therefore, there was no correlation between HCV viral titer changes and changes in the serum albumin, PC, Child score, performance score, ascites grade or hematemesis attacks. However, there was a moderate negative correlation between HCV titer changes with changes in serum bilirubin in the 3rd month only after stem cell transplantation.

Similarly, there was a negative correlation between HCV titer changes and changes in AST in the 3rd and 6th months only after stem cell transplantation, and a moderate negative correlation between HCV titer changes with changes in ALT in the 2nd month only after stem cell transplantation. These results agree with those of Duvoux *et al*[17], who demonstrated that the severity of liver disease is independent of serum levels of HCV.

From the data presented in the current study, which included the largest cohort of HCV-associated end stage liver disease patients to date, we conclude that autologous stem cell transplantation may be safely administered and appears to offer some therapeutic benefit to patients with viral hepatic end-stage liver disease.

ACKNOWLEDGMENTS

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COMMENTS

Background

Stem cell therapy is an innovation technique with promising application in various human diseases. Stem cell plasticity has enabled different researchers to use them in treating different diseases. Recent success using hematopoietic stem cells to support the failing liver in humans has been reported. Stem cells support of the failing liver might reduce or obviate the need for future liver transplantation.

Research frontiers

Stem cells are present in different tissues, with a rich source in the bone marrow. Different manipulation techniques, such stimulation with as granulocyte colony stimulating factor, enabled the scientists to use stem cells effectively to treat different diseases with different etiologies. Due to the high prevalence of hepatitis viruses, such as hepatitis B virus and hepatitis C virus, the proportion of patients with liver cirrhosis and failing liver is increasing linearly. Although liver transplantation is the definitive treatment, its complicated technique, high costs, immune suppression and limited organ supply are major limitations for its wide use. Stem cell therapy is an innovative technique that can offer an effective, safe, and cheap support for patients with failing livers.

Innovations and breakthroughs

Recent reports have highlighted the importance of using stem cells in supporting the failing liver, with promising results for a wide range of etiologies of liver insult. This is the first large, randomized, and controlled study to report the value of using autologous hematopoietic CD34, CD133 stem cells to support the failing liver, with promising 6 mo follow-up results.

Applications

This technique exploits the plasticity of stem cells and the ability of hematopoietic stem cells to change into hepatocytes to support the failing liver in patients with end-stage liver disease of various etiologies. In the future this technique might reduce the need for liver transplantation.

Terminology

Hematopoietic CD34, CD133 Stem cells are bone marrow cells specialized in forming different blood elements and being stem cells, they have the character of plasticity and can change into hepatocytes. Previous human studies and the present one demonstrated, directly and indirectly, the ability of these cells to support the failing liver.

Peer review

In this manuscript, Salama *et al* evaluated the therapeutic efficacy of autologous stem cell transplantation in end-stage liver cirrhosis. They evaluated a large number of patients, and found beneficial effects of this therapy. The issue is not novel, but the current study enrolled the largest series of patients ever evaluated. The study design is fair, and the study is well performed. Findings are sound and of potential impact in the field.

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ORIGINAL ARTICLE

Total salvianolic acid improves ischemia-reperfusion-induced microcirculatory disturbance in rat mesentery

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Abstract

AIM: To investigate the effect of total salvianolic acid (TSA) on ischemia-reperfusion (I/R)-induced rat mesenteric microcirculatory dysfunctions.

METHODS: Male Wistar rats were randomly distributed into 5 groups (n = 6 each): Sham group and I/R group (infused with saline), TSA group, TSA + I/R group and I/R + TSA group (infused with TSA, 5 mg/kg per hour). Mesenteric I/R were conducted by a ligation of the mesenteric artery and vein (10 min) and subsequent release of the occlusion. TSA was continuously infused either starting from 10 min before the ischemia or 10 min after reperfusion. Changes in mesenteric microcirculatory variables, including diameter of venule, velocity of red blood cells in venule, leukocyte adhesion, free radicals released from venule, albumin leakage and mast cell degranulation, were observed through an inverted intravital microscope. Meanwhile, the expression of adhesion molecules CD11b/CD18 on neutrophils was evaluated by flow cytometry. Ultrastructural evidence of mesenteric venules damage was assessed after microcirculation observation.

RESULTS: I/R led to multiple responses in mesenteric post-capillary venules, including a significant increase in the adhesion of leukocytes, production of oxygen radicals in the venular wall, albumin efflux and enhanced mast cell degranulation *in vivo*. All the I/R-induced manifestations were significantly reduced by pre- or post-treatment with TSA, with the exception that the I/R-induced increase in mast cell degranulation was inhibited only by pre-treatment with TSA. Moreover, pre- or post-treatment with TSA significantly attenuated the expression of CD11b/CD18 on neutrophils, reducing the increase in the number of caveolae in the endothelial cells of mesentery post-capillary venules induced by I/R.

CONCLUSION: The results demonstrated that TSA protects from and ameliorates the microcirculation disturbance induced by I/R, which was associated with TSA inhibiting the production of oxygen-free radicals in the venular wall and the expression of CD11b/CD18 on neutrophils.

Key words: *Salvia miltiorrhiza*; Leukocyte adherence; Oxygen-free radicals; Albumin leakage; Ischemia-reperfusion

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Wang MX, Liu YY, Hu BH, Wei XH, Chang X, Sun K, Fan JY, Liao FL, Wang CS, Zheng J, Han JY. Total salvianolic acid improves ischemia-reperfusion-induced microcirculatory disturbance in rat mesentery. *World J Gastroenterol* 2010; 16(42): 5306-5316 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i42/5306.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i42.5306

INTRODUCTION

Ischemia-reperfusion (I/R) injury occurs in a wide range of situations, including trauma, vascular reflow after contraction, percutaneous transluminal coronary angioplasty, thrombolysis treatment, organ transplantation, and hypovolemic shock with resuscitation[1], leading to systemic disorders with high morbidity and mortality. I/R-induced microcirculatory disturbance is the pathological basis of I/R injury, manifesting as a complex process that includes endothelial cell dysfunction, enhanced adhesion of leukocyte, macromolecular efflux, production of reactive oxygen species, and mast cell degranulation[2]. The insults triggered by I/R in microcirculation interplay with each other in various ways that creates a vicious circle that exaggerates the impact, among which, the explosively produced peroxides injure the vessels[2], promote expression of intercellular cell adhesion molecule-1 (ICAM-1)[3] and leukocyte adhesion molecules CD11b/CD18[4], leading to an increase in the adhesion of leukocytes to venules[5]. On the other hand, the leukocytes adhered to the venular wall, in turn, release peroxides and protease[6-9] that injure the endothelium and basement membrane of vessels, causing the leakage of serum[2]. Besides, the degranulated mast cell induced by I/R[2] release pro-inflammatory factors and vasoactive substances to attack the vessels from outside[8-10]. Clearly, an agent that targets the multiple insults simultaneously would be appealing for management of I/R-induced microcirculatory dysfunction. However, the study in this regard remains limited.

Total salvianolic acid (TSA) is the major water-soluble ingredient of *Salvia miltiorrhiza* (SM), including 3,4-dihydroxy-phenyl lactic acid (DLA), salvianolic acid A (Sal A), salvianolic acid B (Sal B) and other salvianolic acids[2]. SM and SM-containing preparations have been clinically used in the prevention and treatment of various vascular diseases[2]. Previous studies have shown that pre-treatment with Cardiotonic Pills (CP, the major ingredients of which are DLA and Sal B) attenuated the gut I/R-induced leukocyte adhesion in liver sinusoids and plasma tumor necrosis factor-α (TNF-α) and endotoxin[11], diminished the thrombi induced by photochemical stimulation in rat mesenteric venules[12], and prevented I/R-induced myocardial microcirculatory disturbance and myocardial damage in rats[13]. DLA attenuated the production of oxygen radicals and the expression of leukocyte adhesion molecules CD11b/CD18 in mesenteric microcirculatory disturbance induced by I/R[14]. Sal A has the potential of anti-oxidation[15-19], and may normalize membrane permeability[19]. Sal B could inhibit endotoxin-induced rat mesenteric microcirculation disturbance, inhibit the expression of adhesion molecule CD11b/CD18, production of hydrogen peroxide and negative oxygen anion[20-22]. However, the extraction process of DLA and Sal B is complex and the extraction conditions are hard to control, leading to a high cost for DLA and Sal B production. On the other hand, TSA, as the major water-soluble ingredient of SM, has the advantage that the extraction procedure is easily manipulated with a high output and low cost. However, whether TSA can improve microcirculatory disturbance induced by I/R is not clear. Therefore, in this study, we investigated the effect of TSA on I/R-induced rat mesenteric microcirculatory dysfunctions.

MATERIALS AND METHODS

Reagents

TSA was purchased from Kun Ming Feng-Shan-Jian Medical Company (Yun-Nan, China). Dihydrorhodamine 123 (DHR) was obtained from Molecular Probes Ltd. (Eugene, OR, USA). Fluorescein isothiocyanate (FITC)-albumin and toluidine blue were obtained from Sigma Chemical Co. (St Louis, MO, USA). FITC-labeled anti-CD11b, FITC-labeled anti-CD18 antibodies were from BD Biosciences Pharmingen (USA), all other chemicals used were of the highest grade available commercially.

Animals

Male Wistar rats, weighing 200-250 g, were obtained from the Animal Center of Peking University Health Science Center (Beijing, certificate No. SCXK 2002-0001), and randomly distributed into Sham group, I/R group, TSA group, TSA + I/R group and I/R + TSA group. The rats were fasted for 12 h before the experiment, allowing for free access to water. All animals were handled according to the guidelines of the Peking University Health Science Center Animal Research Committee, and the surgical procedures and experimental protocol were approved by Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch.

Preparation of rats for I/R

Surgical procedure in the present study was almost the same as our previous study. Rats were anesthetized and the left jugular vein was cannulated for the infusion of TSA or saline. After the abdomen was opened, an ileocecal portion of the mesentery was gently mounted on a transparent plastic stage designed for the rat. The mesentery was kept warm and moist by continuous superfusion with saline solution at 37℃. The mesenteric microcirculation was observed by a transillumination method using an inverted microscope (DM-IRB, Leica, Germany). The mesentery was transilluminated with a 12-V, 100-W, direct current-stabilized light source. A color video camera (Jk-TU53H, Toshiba, Japan) was mounted

on the microscope, and the image was transmitted onto a monitor (J2118A, TCL, Korea). The images were recorded with a Digital Video Disk (DVD) videocassette recorder (DVR-R25, Malata, China). Single unbranched venules with diameters ranging between 30 and 50 μm and length longer than 200 μm were selected for study[2].

After 10 min of basal observation of the hemodynamics in the rat mesenteric microvasculature, the I/R was accomplished by ligating the feeding branch of the anterior mesenteric artery and the corresponding vein simultaneously with a snare created by 2-0 silk suture for 10 min and subsequent release of the blood flow. Red blood cell velocity in the vessels during the ischemia was not zero because of the possible collateral perfusion to the observed area. Thus both artery and vein were ligated to stop blood supply and induce venule congestion to enhance ischemia. A previous study showed that 10-min ischemia followed by reperfusion was long enough to induce mesenteric microcirculatory disturbance with minimum intestinal tissue injury[5]. Sham-operated rats without I/R were used as control[14].

Administration of TSA

In the I/R group, the vehicle saline (8 mL/kg per hour) was infused *via* the left jugular vein catheter starting from 10 min before the ischemia and sustained until the end of the observation. The animals of the sham-operated group (Sham group) received the same infusion as those in the I/R group. The animals of the TSA group received only TSA (5 mg/kg per hour) until the end of the observation, without I/R surgery. In pre-treatment with TSA group (TSA + I/R group), the TSA (5 mg/kg per hour) was continuously infused *via* the left jugular vein catheter starting from 10 min before ischemia until the end of the observation. In post-treatment with TSA group (I/R + TSA group), the TSA (5 mg/kg per hour) was continuously infused *via* the left jugular vein catheter at the same doses as those in pre-treatment group but starting from 10 min after reperfusion until the end of the observation (Figure 1).

Measurement of microvascular parameters

Venular diameter was measured on the recorded video images at baseline (before ischemia), 1, 10, 20, 30, 40, 50 and 60 min after reperfusion, using Image-Pro Plus 5.0 software (Media Cybernetic, USA). The diameter was presented as the mean of three measurements at one location[23].

The velocity of red blood cells (RBCs) in the venule was recorded at a rate of 1000 frames/s using a high-speed video camera system (Ektapro 1000, San Diego, CA, USA), and the recordings were replayed at a rate of 25 frames/s from the high-speed stored images. RBCs velocity in the venule was measured with Image-Pro Plus 5.0 software at baseline (before ischemia), 1, 10, 20, 30, 40, 50 and 60 min after reperfusion[23].

The number of leukocytes adhered to the venules was determined off-line during play-back of videotaped images. Leukocytes adhered to the venules were identified as cells that attached to the same site for more than

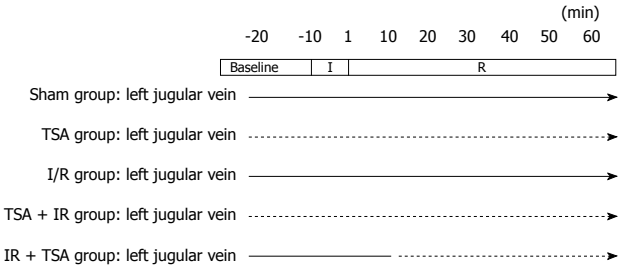

Figure 1 The protocol of the experiment. The saline and total salvianolic acid (TSA) was continuously infused through the left jugular vein in all groups. Solid arrow represents the infusion with saline to the rat; Dotted arrow represents the infusion with TSA to the rat. -20 min represents the time point of 10 min before ischemia; -10 min represents the time point of the beginning of ischemia; 1, 10, 20, 30, 40, 50 and 60 min represent the time point of 1, 10, 20, 30, 40, 50 and 60 min after reperfusion. I: Ischemia; R: Reperfusion.

10 s judging from the replayed video images. The number of adherent leukocytes was counted along venules (30-50 μm in diameter, 200 μm in length) selected from the videotape images recorded and expressed as the number per 200 μm of venule length[23].

The oxidant-sensitive fluorescent probe dihydrorhodamine 123 (DHR; Molecular probes) was added to the mesenteric surface (10 μmol/L) to assess the oxidant stress in venular walls. The excitation light of 455 nm was irradiated from a mercury burner (100 W) to the inverted fluorescence microscope (DM-IRB, Leica, Germany) to observe the fluorescence. The fluorescent image was recorded at baseline (before ischemia), 1, 10, 20, 30, 40, 50 and 60 min after reperfusion, using CD recorder and the fluorescent intensity of venular walls and extravenular interstice was measured with Image-Pro Plus 5.0 software. The difference between the fluorescent intensity of venular wall and extravenular interstice at every time point was determined, and the ratio of the value at each time point to that at baseline was calculated[23].

In another set of experiments, to evaluate the albumin leakage across mesenteric venules, the animals were intravenously injected with 5 mg/kg body weight of FITC-labeled bovine serum albumin. After 10 min of basic observation, an excitation light (455 nm wavelength) was irradiated from a mercury burner (100 W) to the inverted fluorescence microscope (DM-IRB, Leica, Germany). At baseline (before ischemia), 1, 10, 20, 30, 40, 50 and 60 min after reperfusion, the image was recorded using a Compact Disk recorder. The fluorescence intensity of FITC-albumin inside the lumen of selected venules (Iv) and in the surrounding interstitial area (Ii) was estimated. The ratio of Ii/Iv was calculated and compared with the baseline as an indicator of albumin leakage[23].

Sixty minutes after reperfusion, the tissue was stained with 0.1% toluidine blue for 1 min and rinsed with saline. Degranulated mesenteric mast cells were identified by the presence of intracellular granules released into the surrounding tissue, and counted within each circular microscopic field of view with a 20 × objective lens. Five fields were evaluated along the microvasculature for each mes-

enteric window. The numbers of both non-degranulated and degranulated mast cells were scored, and the percentage of degranulated mast cells was thus calculated[23].

Determination of expression of adhesion molecules CD11b and CD18 on neutrophils

In another set of experiments, blood was taken from the abdominal aorta of rats and anticoagulated with heparin, and, afterward, incubated with FITC-labeled anti-CD11b antibody (5 μg/mL) or FITC-labeled anti-CD18 antibody (5 μg/mL) or corresponding FITC-labeled mouse isotype (5 μg/mL) for 20 min at room temperature. The erythrocyte lysis was accomplished using haemolysin according to the manufacturer's instruction, and the cells were washed twice with PBS. The mean fluorescence intensity was calculated with flow cytometry (FACS Calibur; BD Company, USA). Neutrophils were then sorted by characteristic forward-/side-scatter expression as reported previously[23]. Five thousand neutrophils were evaluated for each sample.

Electron microscopy

The mesentery in each experiment condition was also prepared for electron microscopy. Briefly, immediately after 60 min perfusion, the rats, maintained under deep anesthesia, underwent perfusion through the left ventricle with physiological saline followed by 120 mL of phosphate-buffered 40 g/L paraformaldehyde plus 20 g/L glutaraldehyde at a speed of 3 mL/min. The mesentery tissues were then removed, localized and further fixed by immersion in phosphate-buffered 30 g/L glutaraldehyde for 1 h. The tissues were routinely processed for transmission electron microscopy and examined in JEM 1230 (JEOL, Japan)[24].

Statistical analysis

The data were analyzed by one-way ANOVA and Fisher's post test. All values were expressed as mean ± SE of values from 6 rats. $P < 0.05$ was considered statistically significant.

RESULTS

Changes in vascular diameter and velocity of RBC in venules

No significant alteration was observed in the diameter of either arterioles or venules in mesenteric microcirculation during 60 min of observation, and the situation remained unchanged by continuous per- or post-treatment with TSA (data not shown).

I/R induced a significant decrease in the velocity of RBCs over the first 10 min after reperfusion. A similar alteration was observed in the TSA + I/R group and I/R + TSA group. The result demonstrated that pre- or post-treatment with TSA has no significant influence on the decrease of RBCs velocity induced by I/R (data not shown).

Changes in the number of leukocytes adhered to the venular walls

The images of leukocytes adhered to the venular walls in each group at baseline, 10 and 60 min after the reperfusion are illustrated in Figure 2A. No adherent leukocyte was observed before ischemia in each group (a1-e1). At 10 min after reperfusion, a small amount of adherent leukocytes were observed along the venular walls in the animals subjected to I/R (c2-e2). At 60 min after reperfusion, numerous adherent leukocytes were observed along the venular walls (c3) in I/R group. While in TSA+I/R group (d3) and I/R+TSA group (e3), only few of the adherent leukocytes were observed.

The time course of changes in the number of leukocytes adherent to venular walls was examined and the result is depicted in Figure 2B. Obviously, the number of adherent leukocytes in the Sham and TSA group increased only slightly during the whole period of observation. The number of adherent leukocytes was significantly increased from 10 min and further up to 60 min after reperfusion. Pre-treatment with TSA significantly decreased the number of adherent leukocytes at 20 min after reperfusion. Post-treatment with TSA significantly attenuated the further increase of adherent leukocytes from 40 min after reperfusion.

Changes in fluorescence intensity of DHR in the venular walls

The images of fluorescence intensity of DHR in the venular walls in each group at baseline, 10 and 60 min after the reperfusion are illustrated in Figure 3A. No DHR fluorescence was detected on rat mesenteric venular walls before I/R (a1-e1). I/R induced a pronounced DHR fluorescence on mesenteric venular walls (c3). Pre-treatment with TSA significantly attenuated the I/R-induced DHR fluorescence enhancement (d3). Post-treatment with TSA inhibited the I/R-induced increase in DHR fluorescence ratio in a similar fashion (e3).

The time course of changes in DHR fluorescence ratio on the venular walls is presented in Figure 3B. In the Sham group and TSA group, there was no significant change in the DHR fluorescence ratio on the venular wall throughout the observation. In the I/R group, the intensity of DHR fluorescence on the venular wall increased apparently and lineally until the end of reperfusion. Pre-treatment with TSA apparently attenuated the I/R-induced DHR fluorescence intensity on the venular walls at 10 min after reperfusion. Post-treatment with TSA attenuated the further increase of DHR fluorescence intensity on the venular walls at 20 min after reperfusion, with the fluorescence intensity of DHR on the venular wall keeping nearly constant over the remaining time.

Changes in albumin leakage

The images of albumin leakage in each group at baseline, 10 and 60 min after the reperfusion are illustrated in Figure 4A. No albumin leakage was observed before ischemia in all conditions (a1-e1). In the I/R group, apparent leakage was observed at 60 min after reperfusion (c3). Pre-treatment with TSA significantly prevented FITC-labeled albumin leakage from rat mesentery ve-

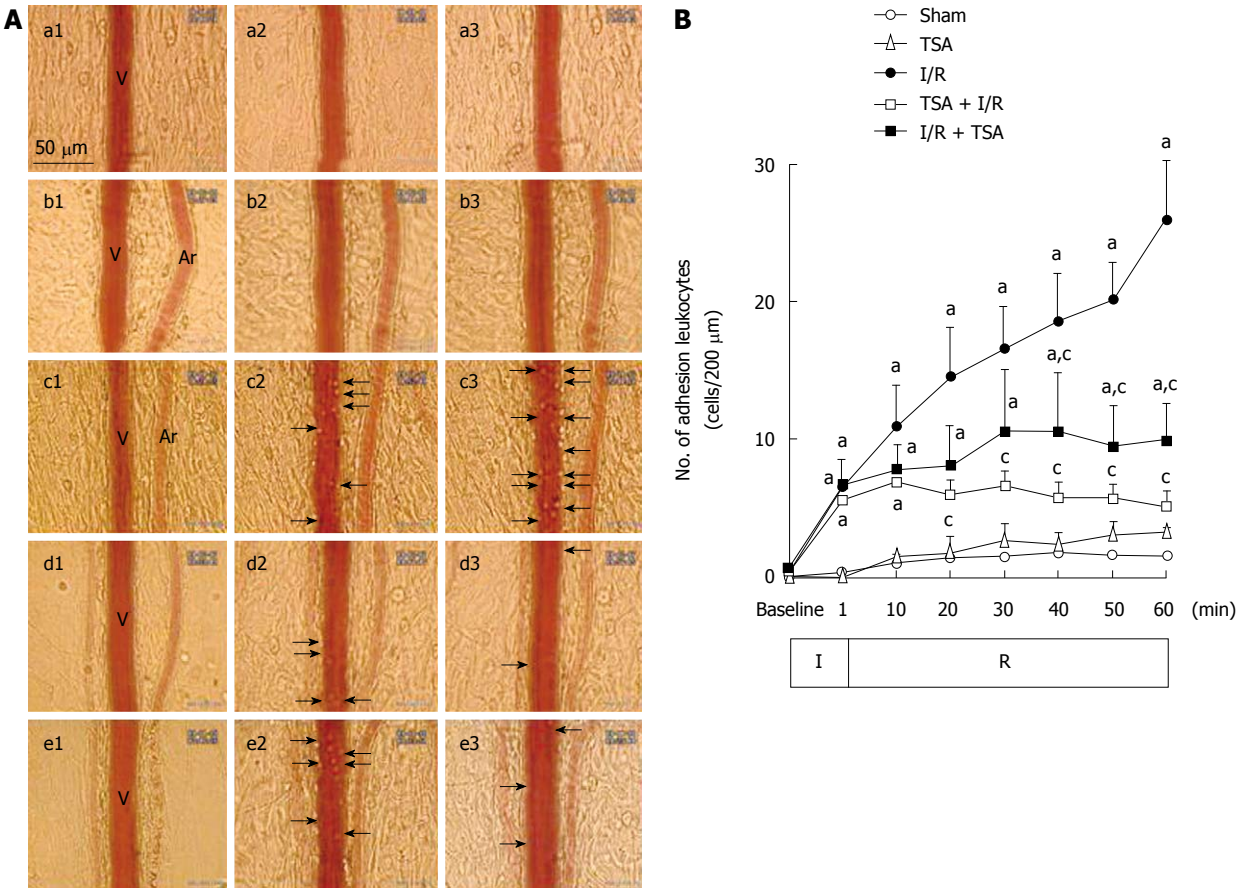

Figure 2 The effect of pre-treatment and post-treatment of total salvianolic acid on ischemia-reperfusion-induced leukocyte adhesion to the rat mesenteric venular wall. A: Representative images illustrating the effect of pre-treatment and post-treatment of total salvianolic acid (TSA) on leukocyte adhesion to the venular wall induced by ischemia-reperfusion (I/R) in rat mesentery. a1-a3: Rat mesentery images of Sham group at baseline, 10 and 60 min, respectively. b1-b3: Rat mesentery images of TSA group at baseline, 10 and 60 min, respectively. c1-c3: Rat mesentery images of I/R group at baseline, 10 and 60 min, respectively. d1-d3: Rat mesentery images of rat mesentery of TSA + I/R group at baseline, 10 and 60 min respectively. e1-e3: Rat mesentery images of I/R + TSA group at baseline, 10 and 60 min, respectively. V: Rat mesenteric venule; Ar: Rat mesenteric arteriole; Arrows: Leukocytes adhered to the venular wall; B: Time course of changes in the number of leukocytes adherent to the mesenteric venules of rat. The number of adherent leukocytes was expressed as the number of cells per 200 μm of venules. Sham: Sham group; TSA: TSA group; I/R: I/R group; TSA + I/R: TSA plus I/R group; I/R + TSA: I/R plus TSA group. Data was expressed as mean ± SE of six animals. $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* I/R alone.

nules challenged by I/R (d3). Post-treatment with TSA prevented further increase in the albumin leakage (e3).

The changes in albumin leakage from the rat mesenteric venules were quantitated and the result is shown in Figure 4B. In the Sham group and TSA group, there was no significant change in the albumin leakage ratio from venular walls throughout the observation. In the I/R group, the albumin leakage from venules increased immediately after the initiation of reperfusion in a time-dependent manner. Pre-treatment with TSA significantly attenuated the albumin leakage from venular walls at 10 min after reperfusion. Post-treatment with TSA attenuated the further increase in the albumin leakage from venular walls from 20 min after reperfusion.

Mast cell degranulation

Mast cell degranulation was examined after 60 min of reperfusion in various conditions (Figure 5A). I/R evoked an apparent increase in mast cell degranulation (c), while pre-treatment with TSA significantly inhibited the mast cell degranulation (d). The results were quantified as the percent of the number of degranulated mast cells to the total number of mast cells examined (Figure 5B). The percentages of degranulated mast cells in the Sham group and TSA group were 20.7% ± 0.7% and 21.7% ± 2.5%, respectively, representing the spontaneous occurrence of the mast cell degranulation in the present case. I/R resulted in an apparent increase in the mast cell degranulation (62.2% ± 2.5%), and this increase was suppressed significantly by pre-treatment with TSA (23.7% ± 4.7%). Post-treatment with TSA almost had no effect on mast cell degranulation compared to I/R group (52.4% ± 2.2% *vs* 62.2% ± 2.5%).

Fluorescence intensity of adhesion molecules CD11b and CD18 in neutrophils

An *in vitro* study was performed to determine the fluorescence intensity of adhesion molecules CD11b and CD18 in neutrophils (Figure 6). The fluorescence intensity of CD11b (Figure 6B) and CD18 (Figure 6A) was significantly increased by I/R compared to the Sham group. Pre-treatment with TSA significantly inhibited the increase in

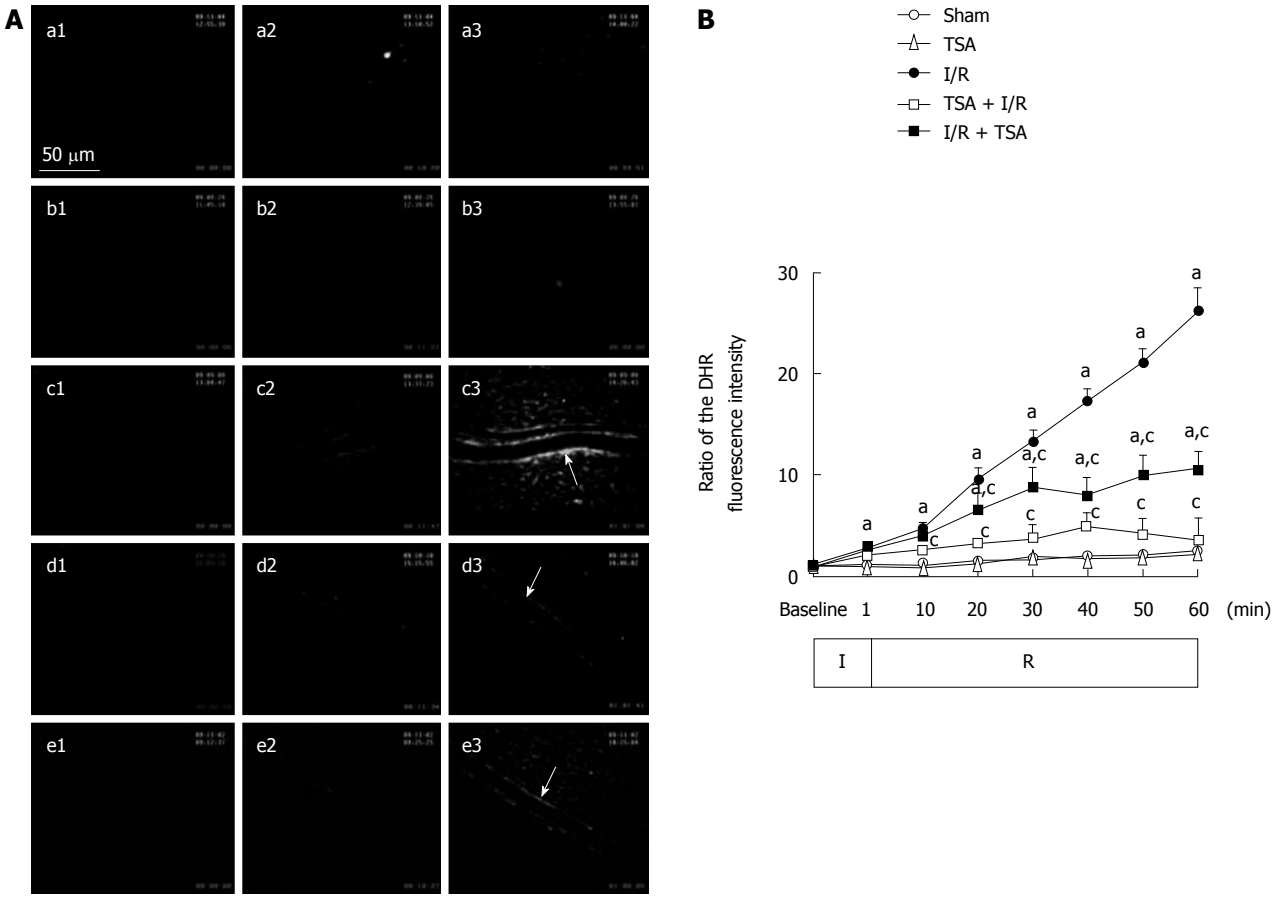

Figure 3 The effect of pre-treatment and post-treatment of total salvianolic acid on ischemia-reperfusion-induced dihydrorhodamine 123 fluorescence in rat mesenteric venular wall. A: Representative images of the changes in fluorescence intensity of the H_2O_2-sensitive probe dihydrorhodamine 123 (DHR) in the rat mesenteric venular wall. a1-a3: DHR fluorescence images of rat mesentery of Sham group at baseline, 10 and 60 min, respectively. b1-b3: Fluorescence images of rat mesentery of total salvianolic acid (TSA) group at baseline, 10 and 60 min, respectively. c1-c3: Fluorescence images of rat mesentery of ischemia-reperfusion (I/R) group at baseline, 10 and 60 min, respectively. d1-d3: Fluorescence images of rat mesentery of TSA + I/R group at baseline, 10 and 60 min, respectively. e1-e3: Fluorescence images of rat mesentery of I/R + TSA group at baseline, 10 and 60 min, respectively. Baseline, no DHR fluorescence is visible in all groups (a1-e1). 60 min after I/R, prominent DHR fluorescence occurs on the wall of venule of rat mesentery (c3-e3). Arrows: DHR fluorescence on the venular wall; B: Time course of changes in DHR fluorescence ratio on the venular walls. Sham: Sham group; TSA: TSA group; I/R: I/R group; TSA + I/R: TSA plus I/R group; I/R + TSA: I/R plus TSA group. Data was expressed as mean ± SE of six animals. $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* I/R alone.

fluorescence intensity of CD11b and CD18 in neutrophils induced by I/R. A similar effect was observed for post-treatment with TSA.

Ultrastructure changes in post-capillary venules

Figure 7 presents the electron micrographs of rat mesentery post-capillary venules in each group. In the Sham group (A) and TSA group (B) venules were lined by a layer of endothelial cells, which exhibited a rather smooth inner face with occasionally occurring vesicles in the cytoplasm. At 10 min after reperfusion (C) in I/R group, the adherent leukocyte and platelet were observed within the lumen of venules in rat mesentery, and numerous caveolae emerged in the endothelial cells of post-capillary venules. At 60 min after reperfusion, an even more apparent alteration in the ultrastructure of the endothelial cell occurred, characterized by the increase in the number and size of caveolae in the cytoplasm (D). I/R-induced alterations in the ultrastructures of endothelial cell were abated by pre-and post-treatment with TSA (E and F).

DISCUSSION

With the animal model of rat mesenteric microcirculation disturbance induced by I/R, this research demonstrated that pre-treatment with TSA has an improvement effect on the adhesion of leukocytes to venules, production of peroxides in the venular wall, albumin leakage and mast cell degranulation induced by I/R. Post-treatment with TSA displays an improvement action on the I/R-induced insults likewise, except for the mast cell degranulation.

The diameters of the mesenteric venules were not changed after I/R, and TSA had no effect on venular diameter during I/R, suggesting that TSA does not affect vascular tone. Therefore, TSA exerts protective effects on I/R-induced microcirculatory disturbance through a way other than vasodilation. In addition, both pre- and post-treatment with TSA had no apparent influence on the decrease of RBCs velocity induced by I/R, indicating that TSA does not affect the hemodynamics of the rats.

One of the interesting results in this study is that pre-

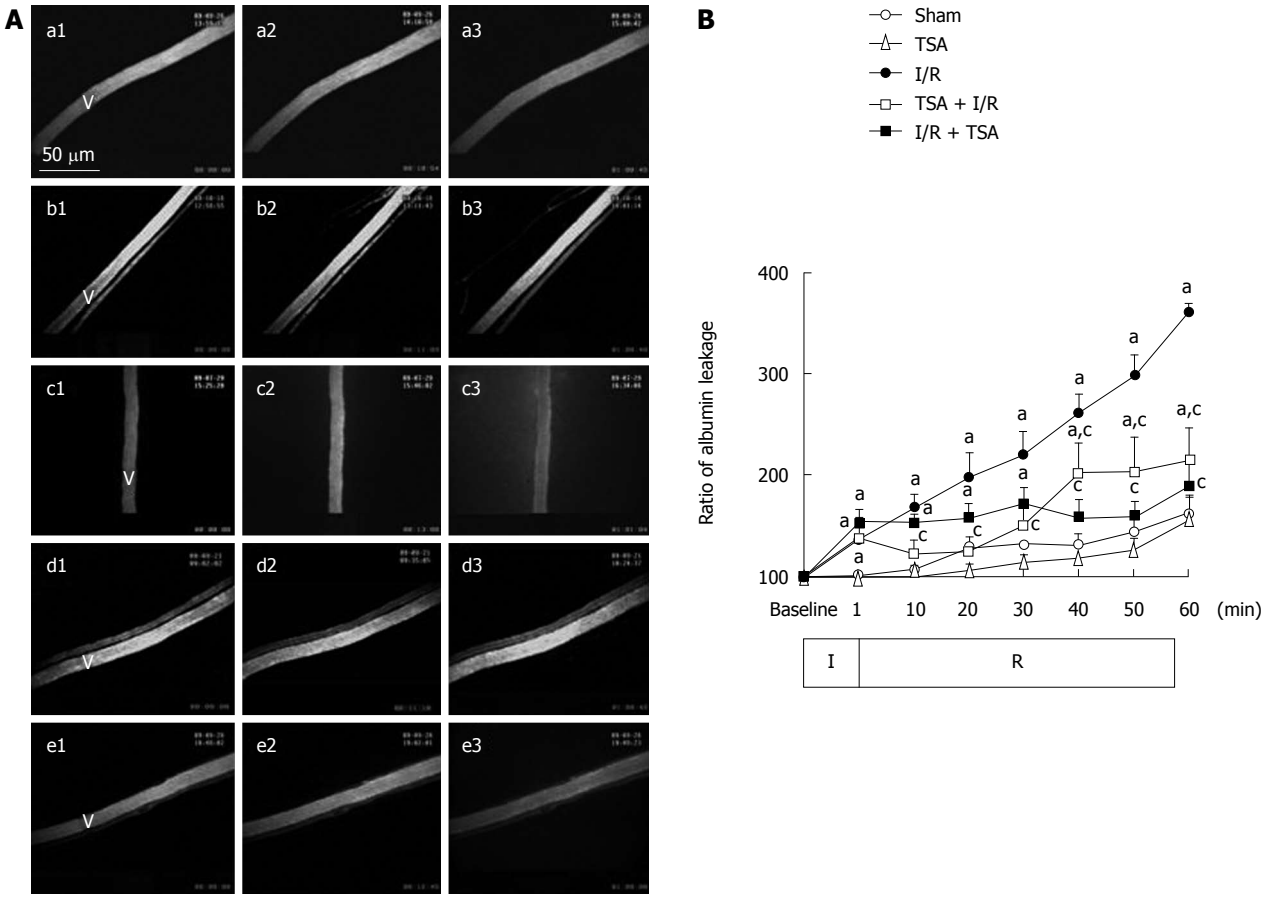

Figure 4 The effect of pre-treatment and post-treatment of total salvianolic acid on ischemia-reperfusion-induced albumin leakage from rat mesenteric venule. A: Representative images for the effect of pre-treatment and post-treatment with total salvianolic acid (TSA) on fluorescein isothiocyanate-labeled albumin leakage from mesentery venule. a1-a3: Fluorescence images of rat mesentery of Sham group at baseline, 10 and 60 min, respectively. b1-b3: Fluorescence images of rat mesentery of TSA group at baseline, 10 and 60 min, respectively. c1-c3: Fluorescence images of rat mesentery of ischemia-reperfusion (I/R) group at baseline, 10 and 60 min, respectively. d1-d3: Fluorescence images of rat mesentery of TSA + I/R group at baseline, 10 and 60 min, respectively. e1-e3: Fluorescence images of rat mesentery of I/R + TSA group at baseline, 10 and 60 min, respectively. V: Rat mesenteric venule; B: Times course of changes in the albumin leakage from mesentery venules. Sham: Sham group; TSA: TSA group; I/R: I/R group; TSA + I/R: TSA plus I/R group; I/R + TSA: I/R plus TSA group. Data was expressed as mean ± SE of six animals. $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* I/R alone.

and post-treatment with TSA inhibited the DHR fluorescence intensity in the venular walls following I/R. It has been reported that I/R challenge leads to production of oxygen-free radicals by xanthine oxidase[25], and leukocyte adhered to vascular walls generates a peroxide burst through the NADPH oxidase[26-29] is one of the reasons of injury in vascular endothelium and basement membrane[30,31]. So inhibition of the production of oxygen-free radical induced by I/R can help protect the blood vessels. Previous studies have shown that DLA can clear the negative oxygen anions produced through the xanthine oxidase system[32-34]. Sal A was reported to inhibit cerebral lipid peroxidation after I/R, and scavenge oxygen-free radical *in vitro*[18]. Sal B could eliminate 1,1-diphenyl-2-picrylhydrazyl (DPPH), inhibit lipid peroxidation[35] and production of reactive oxygen species[36], remove hydrogen peroxide in a dose-dependent way and inhibit the activity of NADPH and peroxide generation induced by TNF-α[37]. However, the effect of TSA on the production of oxygen free radicals on venular walls induced by I/R *in vivo* has not been reported. In the present study, we assessed the production of hydrogen peroxide (H_2O_2) in the venular wall with the aid of DHR, a fluorescence probe that transforms to rhodamine when reacting with hydrogen peroxide, and proved that TSA can inhibit the DHR fluorescence intensity on the venular walls following I/R. The mechanism for TSA to inhibit the hydrogen peroxide remains to be elucidated, and it is most likely that its ingredients DLA, Sal A and Sal B work in coordination through their own pathways to produce a collective outcome.

Another important observation in this study was that pre- or post-treatment with TSA can inhibit I/R induced adhesion of leukocytes to venular walls. It was reported that I/R promotes the expression of ICAM-1 on endothelium[38] and CD11b/CD18 in neutrophils, leading to the adhesion of leukocyte to vascular wall[39,40]. Inhibiting the adhesion of leukocytes to the vascular wall is another important part of improving the microcirculatory disturbance induced by I/R. Our previous study also demonstrated that DLA can inhibit I/R-induced adhesion of leukocyte to rat mesenteric venule and expression of adhesion molecule CD11 b/CD18 *in vitro*[20]. No report has been published so far concerning inhibition of TSA on

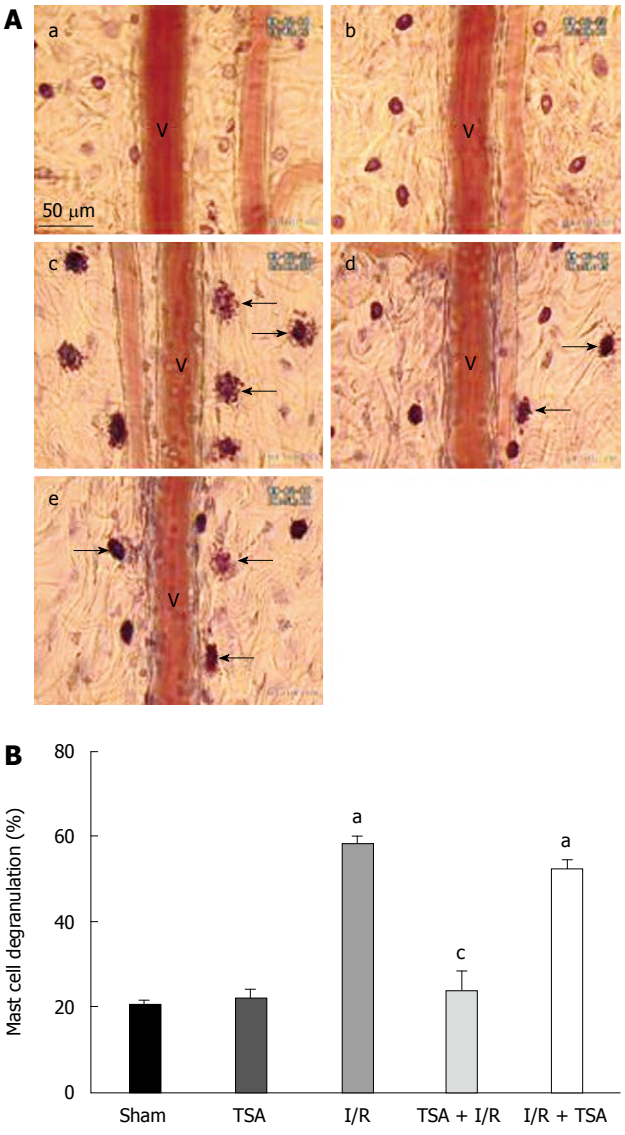

Figure 5 Effect of total salvianolic acid on mast cell degranulation induced by ischemia-reperfusion in rat mesentery. A: Representative image of degranulated mast cells in the mesentery after ischemia-reperfusion (I/R). a: Mast cells of Sham group; b: Mast cells of total salvianolic acid (TSA) group; c: Mast cells of I/R group; d: Mast cells of TSA + I/R group; e: Mast cells of I/R + TSA group. V: Rat mesenteric venule; Arrows: degranulated mast cells. B: A quantitative evaluation of mast cell degranulation along venules in Sham group, TSA group, I/R group, TSA + I/R group, I/R + TSA group. Data was expressed as mean ± SE of six animals. $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* I/R alone.

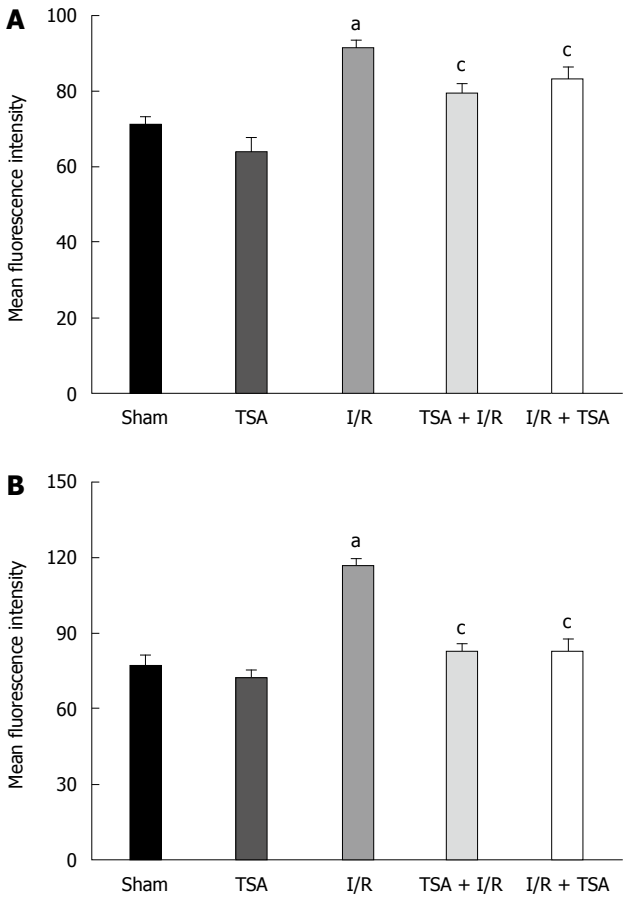

Figure 6 Effect of total salvianolic acid on ischemia-reperfusion-induced expression of adhesion molecules CD18 (A) and CD11b (B) on rat neutrophils. The expression of adhesion molecules is presented as fluorescence intensity on ordinate. Data was expressed as mean ± SE of six animals. $^{a}P < 0.05$ *vs* sham group; $^{c}P < 0.05$ *vs* ischemia-reperfusion (I/R) alone.

leukocyte adhesion to venular walls induced by I/R. This study proved that pre- or post-treatment with TSA can inhibit I/R induced adhesion of leukocytes to venular walls, which was associated with the inhibition of TSA on the expression of CD11b/CD18 in neutrophils.

I/R induced mast cell degranulation, resulting in release of pro-inflammatory factors[41,42] and vasoactive substances that attack the vessels from outside, increasing the vascular damage. Inhibiting the degranulation of mast cells after I/R can reduce the attacks on vessels from outside. This study proved that pre-treatment with TSA can inhibit I/R induced mast cell degranulation. However, based on the result that post-treatment with TSA could not inhibit mast cells degranulation, the inhibitory effect of TSA upon mast cells degranulation is considered to be an indirect result.

In the present study, we demonstrated that pre- or post-treatment with TSA could inhibit FITC-albumin leakage from venules. It was documented in the lung[43] and myocardium[44] that the increased vascular permeability induced by inflammatory stimulations is due to the enlarged population of caveolae, which play a significant role in microvascular permeability[44]. In line with these findings, the result of electron microscopy in the present study showed that the number of caveolae in the endothelial cells of venules was apparently increased at 60 min after reperfusion, whereas the endothelial cells themselves and the intercellular junctions remained intact, implying that I/R-induced albumin leakage observed in the present situation was mainly accomplished by the caveola. The present study demonstrated that pre- or post-treatment with TSA could reduce the number of endothelial caveola as well as FITC-labeled albumin leakage from venular wall induced by I/R, suggesting that it inhibited albumin leakage from venular wall by modulating the number of endothelial caveola. In addition, the inhibition of TSA on FITC-albumin leakage may be also related to inhibition of peroxide generation and the adhesion of leukocytes to venules, thus protecting the venule from inside.

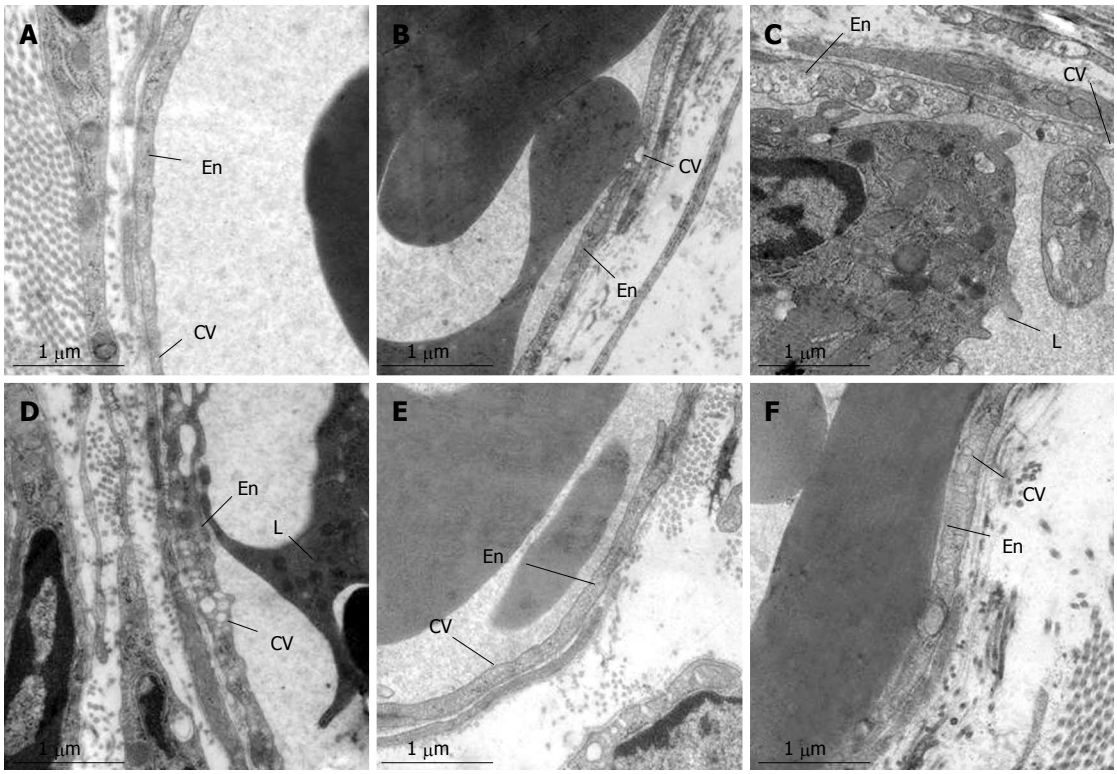

Figure 7 Representative electron micrographs of post-capillary venules of rat mesentery. A: Sham group; B: Total salvianolic acid (TSA) group; C: 10 min after reperfusion; D: 60 min after reperfusion; E: TSA + ischemia-reperfusion (I/R) group; F: I/R + TSA group. En: Endothelial cell; CV: Caveolae; L: Leukocyte.

In summary, the present study demonstrated that pretreatment with TSA significantly improved the microcirculatory dysfunction in rat mesentery induced by I/R. TSA post-treatment starting from 20-min reperfusion ameliorated I/R-induced microcirculatory disorders in rat mesentery alike but in a distinct manner. The *in vitro* experiment revealed that H_2O_2-elicited enhancement in the expression of CD11b and CD18 on neutrophils was significantly attenuated by treatment with TSA, raising the possibility that the ability of TSA to inhibit the leukocyte adhesion may be correlated with its potential to suppress the expression of adhesion molecules. It is likely that the antioxidant activity of TSA and its inhibition on the expression of adhesion molecules CD11b/CD18 on leukocyte are underlying its ameliorating action on I/R-induced microcirculatory disturbance in rat mesentery.

COMMENTS

Background

Ischemia-reperfusion (I/R) injury occurs in a wide range of situations, including trauma, vascular reflow after contraction, percutaneous transluminal coronary angioplasty, thrombolysis treatment, organ transplantation, and hypovolemic shock with resuscitation. I/R-induced microcirculatory disturbance with diverse manifestations is considered to be the pathological basis of I/R injury. An agent that targets the multiple insults simultaneously would be appealing for management of I/R-induced microcirculatory dysfunction. However, the study in this regard remains limited. Total salvianolic acid (TSA) is the major water-soluble ingredient of Salvia miltiorrhiza (SM), SM and SM-containing preparations have been clinically used in the prevention and treatment of various vascular diseases. However, whether the TSA can improve microcirculatory disturbance induced by I/R are not clear. The results of the present study provide evidence for the beneficial role of TSA in attenuating microcirculatory disorders, using I/R-induced rat mesenteric microcirculatory dysfunction as a model.

Research frontiers

3,4-dihydroxy-phenyl lactic acid, salvianolic acid A and salvianolic acid B are the main ingredients of TSA. However, the extraction process of 3,4-dihydroxy-phenyl lactic acid, salvianolic acid A and salvianolic acid B is complex and the extraction conditions are hard to control. On the other hand, TSA, if taking as a whole, has the advantage of high output and low cost in preparation. The present work shows the potential of TSA for management of I/R-induced microcirculatory dysfunctions. Innovations and breakthroughs: this study proves, for the first time, the ability of TSA to inhibit I/R-induced dihydrorhodamine 123 fluorescence intensity, and adhesion of leukocytes to venular walls, the latter of which was possibly associated with the inhibition of TSA on the expression of CD11b/CD18 in neutrophils.

Applications

These results provide a theoretical support for TSA containing Chinese medicine intervention in microcirculatory disturbance induced by thrombolytic therapy or other I/R-related clinical events.

Terminology

TSA is the water-soluble extract of SM, consisting of 3,4-dihydroxy-phenyl lactic acid, salvianolic acid A and salvianolic acid B as major ingredients.

Peer review

The authors demonstrated the inhibitory effect of total salvianolic acid inhibiting several markers associated with ischemia-reperfusion injury. This paper was well-organized and well-investigated, and has a novel finding in this manuscript.

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BRIEF ARTICLE

Antibiotic prophylaxis in variceal hemorrhage: Timing, effectiveness and *Clostridium difficile* rates

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Abstract

AIM: To investigate if antibiotics administered within 8 h of endoscopy reduce mortality or increase the incidence of *Clostridium difficile* infection (CDI).

METHODS: A 2-year retrospective analysis of all patients who presented with first variceal hemorrhage was undertaken. The primary outcome measure was 28-d mortality. Secondary outcome measures were 28-d rebleeding rates and 28-d incidence of CDI. All patients were admitted to a tertiary liver unit with a consultant-led, 24-h endoscopy service. Patients received standard care including terlipressin therapy. Data collection included: primary and secondary outcome measures, timing of first administration of intravenous antibiotics, etiology of liver disease, demographics, endoscopy details and complications. A prospective study was undertaken to determine the incidence of CDI in the study population and general medical inpatients admitted for antibiotic therapy of at least 5 d duration. Statistical analysis was undertaken using univariate, non-parametric tests and multivariate logistic regression analysis.

RESULTS: There were 70 first presentations of variceal hemorrhage during the study period. Seventy percent of cases were male and 65.7% were due to chronic alcoholic liver disease. In total, 64/70 (91.4%) patients received antibiotics as prophylaxis during their admission. Specifically, 53/70 (75.7%) received antibiotics either before endoscopy or within 8 h of endoscopy [peri-endoscopy (8 h) group], whereas 17/70 (24.3%) received antibiotics at > 8 h after endoscopy or not at all (non peri-endoscopy group). Overall mortality and rebleeding rates were 13/70 (18.6%) and 14/70 (20%), respectively. The peri-endoscopy (8 h) group was significantly less likely to die compared with the non peri-endoscopy group [13.2% *vs* 35.3%, $P = 0.04$, odds ratio (OR) = 0.28 (0.078-0.997)] and showed a trend towards reduced rebleeding [17.0% *vs* 29.4%, $P = 0.27$, OR = 0.49 (0.14-1.74)]. On univariate analysis, the non peri-endoscopy group [$P = 0.02$, OR = 3.58 (1.00-12.81)], higher model for end-stage liver disease (MELD) score ($P = 0.02$), presence of hepatorenal syndrome [$P < 0.01$, OR = 11.25 (2.24-56.42)] and suffering a clinical episode of sepsis [$P = 0.03$, OR = 4.03 (1.11-14.58)] were significant predictors of death at 28 d. On multivariate logistic regression analysis, lower MELD score [$P = 0.01$, OR = 1.16 (1.04-1.28)] and peri-endoscopy (8 h) group [$P = 0.01$, OR = 0.15 (0.03-0.68)] were independent predictors of survival at 28 d. The CDI incidence (5.7%) was comparable to that in the general medical population (5%).

CONCLUSION: Antibiotics administered up to 8 h following endoscopy were associated with improved survival at 28 d. CDI incidence was comparable to that in other patient groups.

Key words: Variceal hemorrhage; Mortality; Antibiotics; Prophylaxis; *Clostridium difficile*

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INTRODUCTION

In chronic liver disease (CLD), variceal bleeding occurs in 22%-61% of patients with cirrhosis during study follow-up periods of 12-42 mo[1-5]. Seventy percent of bleeding occurs in the 2 years following diagnosis[6]. Historical data suggest a mortality at the time of first and subsequent variceal hemorrhage of 24%-49% and 30%, respectively[7]. Following variceal hemorrhage, higher mortality is associated with increasing severity of CLD as assessed by both the Child-Pugh score (mean survival 37.3 mo with score A versus 11 mo with score C)[8] and Model for End-stage Liver Disease (MELD) score[9]. More recently, American and European data have shown that 6-wk mortality rates have fallen, ranging from 14% to 20%[9-12], and following a UK-wide audit, a 28-d mortality figure of 14% for first and subsequent variceal bleeds has been quoted[13].

A number of factors are associated with poor outcome following variceal hemorrhage[10], including MELD score, transfusion requirement, alcohol as etiology, bilirubin, albumin, hepatic encephalopathy and hepatocellular carcinoma[9,10]. Higher hepatic venous pressure gradient at the time of variceal hemorrhage predicts length of stay, greater transfusion requirement and death[14].

There are several clinical scoring tools available to predict outcome, rebleeding and need for endoscopic intervention following acute gastrointestinal hemorrhage, including the Rockall and Blatchford scores[15,16]. However, scoring tools for predicting outcome following variceal hemorrhage are not commonly used due to difficulty in the identification of predictive variables. A recent publication has shown that severity of liver disease, renal impairment and infection adversely affect outcome following variceal hemorrhage[17].

Bacterial infection is commonly associated with variceal hemorrhage and appears to be an independent risk factor for failure to control bleeding[18] and predicts both early rebleeding and death[19,20]. The routine use of prophylactic broad-spectrum antibiotics has shown a marked improvement in outcome in acute variceal hemorrhage. Routine intravenous ceftriaxone or post-endoscopic norfloxacin reduces rebleeding rates compared to on-demand antibiotics[21,22]. A Cochrane meta-analysis of antibiotic prophylaxis in cirrhotic patients with gastrointestinal bleeding, given either before and after variceal hemorrhage, revealed a 27% reduction in mortality and a reduction in the incidence of bacterial infections by 60%[23].

United States guidelines for antibiotic prophylaxis in cirrhotic patients admitted with upper gastrointestinal hemorrhage have been published by the American Society of Gastrointestinal Endoscopy, and recommend intravenous ceftriaxone on admission as first-line prophylaxis[24].

The British Society of Gastroenterology has recently updated guidelines on the use of antibiotic prophylaxis in gastrointestinal endoscopy. The recommendation is to administer a ureidopenicillin or third-generation cephalosporin to all patients with suspected variceal bleeding, or those with decompensated liver disease who develop gastrointestinal bleeding, prior to endoscopy[25].

In the Cochrane primary studies, antibiotics were given either before or after endoscopy, therefore, the evidence for when antibiotics should be given remains unclear. Of the studies using post-endoscopic antibiotics, the timing of antibiotic administration has rarely been reported. Indeed, the Cochrane review has acknowledged that evidence for the timing of administration of antibiotics is lacking[23].

The use of broad-spectrum antibiotics raises concerns regarding healthcare-associated infections, particularly *Clostridium difficile* (*C. difficile*) infection (CDI). In the United States, the number of hospital admissions due to CDI has steadily risen over the last decade from 2.7 per 1000 admissions in 2000 to 5.1 per 1000 in 2003, with the highest incidence in patients > 65 years (228 per 100 000 patient-years)[26]. A similar picture has been seen in England until recently[27,28], with 80% of cases occurring in the > 65-year-old population[28,29].

A recent study has shown that CDI in cirrhotic patients is associated with a higher mortality compared to cirrhotic patients without CDI[30]. There is currently no literature on the incidence of CDI in cirrhotic patients admitted with acute variceal hemorrhage who are given prophylactic broad-spectrum antibiotics.

The aims of this study were to determine if prophylactic antibiotics, or more accurately peri-endoscopy antibiotics (administered before, during or up to 8 h following endoscopy), were effective in reducing mortality following first variceal hemorrhage, and to assess the rebleeding rates and incidence of CDI infection in this patient population.

MATERIALS AND METHODS

Patient population

This was a retrospective analysis of cases of first variceal hemorrhage who presented to the Southampton Liver Unit, a tertiary referral centre, from December 1, 2006 to December 1, 2008. Seventy cases were identified from the endoscopy computer database using multiple search strategies. Only first presentation variceal hemorrhages were included. All cases of variceal hemorrhage transferred to the Southampton liver unit from other centers were excluded from the analysis.

All patients were admitted to the liver unit after admission and were treated under the care of a consultant hepatologist and received standard care, including the use of intravenous vasoactive drugs, access to interventional radiology and a 24-h on-call therapeutic endoscopy service. A protocol for the dose and duration of the terlipressin prescription was followed in all cases.

Data collection

Data collection included demographics, etiology of CLD, antibiotic therapy (timing of first dose relative to the endoscopy, type of antibiotic prescribed and duration of therapy), endoscopy details, terlipressin use (dose and duration of therapy), rebleeding rates within 28 d, survival at 28 d, incidence of CDI at 28 d, incidence of sepsis, hepatorenal syndrome, spontaneous bacterial peritonitis and ascites. The data were collected from hospital notes and computerized pathology databases. The microbiology database collected CDI cases from the community and therefore cases occurring after discharge form hospital were also included.

All patients who received antibiotics within the a priori time period of before, at the time of, or within the 8 h following endoscopy were included in the first analysis group [peri-endoscopy (8 h) group]. This group was compared to the second patient group, those patients receiving antibiotics > 8 h after endoscopy or not receiving antibiotics (non peri-endoscopy group).

CDI incidence

To compare the incidence of CDI in cirrhotic patients with general medical patients receiving antibiotics, an additional prospective study was undertaken during the variceal study period. Over one calendar month, all general medical patients admitted for antibiotic therapy of at least 5 d duration for non-gastrointestinal reasons were studied on two general medical wards. All patients were < 80 years of age. Patients were excluded if there was a history of prior CDI infection.

Statistics analysis

To compare demographic data between survivors and non-survivors at 28 d, initial assessments for normality of data distribution were undertaken using the Shapiro-Wilk test and Q-Q plot. The data were found to be non-normally distributed (for example MELD score data has a Shapiro-Wilk P value < 0.01 for normality and a sigmoidal curve on Q-Q plot). As a consequence, all further statistical analyses were undertaken using non-parametric tests.

Initially, univariate analysis was performed on ordinal data using the χ^2 test to compare outcome (mortality, rebleeding or CDI, the dependent variable) with each risk factor (antibiotics within 8 h, hepatorenal syndrome and sepsis, the independent variable). Continuous demographic data (age, INR, creatinine, bilirubin, sodium and MELD score) were compared between survivors and non-survivors using the Mann-Whitney U test.

Following univariate analysis, a forward multivariate logistic regression model was fitted with all statistically significant variables on univariate analysis. However, given the sample size and number of events, only two variables were fitted to the model. The first was the initial study variable, the administration of antibiotics within 8 h of endoscopy, and the second was the MELD score assessment of severity of CLD. All statistical analyses were performed using SPSS (version 16) and STATA 11 software.

Table 1 Baseline demographics and clinical characteristics of patients (mean ± SD) *n* (%)

Parameter	Alive (n = 57)	Dead (n = 13)	Total (n = 70)
Sex (M:F)	38:19 (66.7)	11:2 (84.6)	49:21 (70.0)
Age (yr)	52.93 ± 13.07	51.46 ± 10.52	52.66 ± 12.65
Etiology			
ALD	37 (64.9)	9 (69.2)	46 (65.7)
HCV	1 (1.8)	0 (0)	1 (1.4)
MISC	8 (14.0)	3 (23.1)	11 (15.7)
DUAL	6 (10.5)	1 (7.7)	7 (10.0)
NASH	5 (8.8)	0 (0)	5 (7.1)
INR	1.58 ± 0.47	1.78 ± 0.66	1.62 ± 0.52
Creatinine	86.23 ± 33.87	99.38 ± 42.28	88.67 ± 35.95
Bilirubin	86.74 ± 95.89	190.23 ± 158.96	105.96 ± 117.47[a]
Sodium	136.89 ± 5.46	135.23 ± 6.10	136.59 ± 5.62
MELD	14.79 ± 6.76	20.38 ± 7.58	15.83 ± 7.25[a]

[a]P < 0.05. ALD: Alcoholic liver disease; HCV: Hepatitis C viral infection; MISC: Miscellaneous; DUAL: Dual aetiology; NASH: Non-alcoholic steatohepatitis; INR: International normalised ratio; MELD: Model for end-stage liver disease.

RESULTS

Patients

Between December 1, 2006 and December 1, 2008, 70 patients with first presentation variceal hemorrhage were admitted to the liver unit, Southampton General Hospital. Baseline demographics are shown in Table 1. The majority of patients were male (70%), and the most common etiology of cirrhosis was alcohol (65.7%). Patients who died within 28 d of the first variceal hemorrhage had a higher baseline MELD score (P = 0.02), mainly due to a higher admission serum bilirubin level (Table 1, P = 0.01). The other component parameters of the MELD score were similar in both groups.

Sixty-four (91.4%) patients received prophylactic antibiotics during their admission, with median (inter-quartile range; IQR) and mean (SE) times from endoscopy to administration of antibiotics of 3.5 (0-7.0) and 5.9 (1.18) h, respectively. The most common antibiotic prescription was cefuroxime and metronidazole (n = 45), followed by ciprofloxacin alone (n = 8) and cefuroxime alone (n = 7). The remaining patients received meropenem alone, or vancomycin in combination with either metronidazole or gentamicin. The median (IQR) and mean (SE) duration of antibiotic therapy was 5.0 (3.0-7.0) and 5.7 (0.57) d, respectively.

Twenty-one patients received antibiotics before or at the time of endoscopy and a further 32 received antibiotics after but within 8 h of the index endoscopy. Therefore, a total of 53 (75.7%) patients received antibiotics before,

Table 2 Univariate analysis for 28-d outcome following first variceal hemorrhage

Variable	Mortality at 28 d			Rebleeding at 28 d		
	P value	OR	95% CI	*P* value	OR	95% CI
MELD	0.02	-	-	0.20	-	-
Antibiotics within 8 h (peri-endoscopy)	0.04	0.279	0.078-0.997	0.27	0.491	0.138-1.741
Hepatorenal failure	< 0.01	11.25	2.243-56.421	0.71	1.389	0.249-7.755
Sepsis	0.03	4.029	1.113-14.581	0.89	0.902	0.218-3.730

MELD: Model for end-stage liver disease; OR: Odds ratio; CI: Confidence interval.

during or within 8 h of endoscopy, and were included in the peri-endoscopy (8 h) group.

Of the 17 (24.3%) patients in the non peri-endoscopy group, 11 received antibiotics at > 8 h after endoscopy and six did not receive antibiotic prophylaxis.

The vast majority of patients received endoscopic variceal band ligation therapy during the index endoscopy (n = 54) with only four receiving sclerotherapy. Nine patients required a Sengstaken-Blakemore tube. Eight patients received transjugular intrahepatic portosystemic shunting during their admission. None of these factors were significantly associated with mortality, although banding therapy significantly reduced the incidence of rebleeding [P = 0.01, odds ratio (OR) = 0.191, 95% confidence interval (95% CI) = 0.054-0.680], and the requirement for a Sengstaken-Blakemore tube was significantly associated with rebleeding (P < 0.01, OR = 27, 95% CI = 4.66-156.57). Nine patients failed endoscopic therapy and, while there was a trend towards increased mortality in this group, it was not statistically significantly (P = 0.22, OR = 2.55, 95% CI = 0.545-11.928).

Clinical outcomes

At day 28 following first variceal hemorrhage, 13 patients (18.6%) had died. The peri-endoscopy (8 h) group showed a significant survival benefit when compared to the non peri-endoscopy group. Antibiotics given before, during or within 8 h significantly reduced mortality at day 28 (P = 0.04, OR = 0.279, 95% CI = 0.078-0.997) and showed a trend towards reducing 28-d rebleeding (P = 0.27, OR = 0.491, 95% CI = 0.138-1.741).

CDI incidence

Four patients (5.7%) with first variceal hemorrhage developed CDI within 28 d of admission. All episodes of CDI were in patients who survived at least 28 d post-variceal hemorrhage. CDI did not predict death (P = 0.33) or rebleeding (P = 0.80). During the study period, the prospective study of patients under 80 years of age admitted to one of two general medical wards in Southampton General Hospital for at least 5 d of broad-spectrum systemic antibiotics were monitored. Of the 40 cases identified, two (5%) developed CDI.

Predictors of survival following first variceal hemorrhage

Univariate analysis revealed four risk factors that were significantly associated with death at 28 d (Table 2): receiving antibiotics > 8 h after endoscopy (P = 0.04, OR = 3.584, 95% CI = 1.003-12.808); higher MELD score (P = 0.02); presence of hepatorenal syndrome (P < 0.01, OR = 11.25, 95% CI = 2.243-56.421); and an episode of clinical sepsis (P = 0.03, OR = 4.029, 95% CI = 1.113-14.583). A forward multivariate logistic regression model was undertaken for 28-d survival. A lower MELD score (P = 0.01, OR = 1.155, 95% CI = 1.041-1.281) and administration of antibiotics within 8 h (peri-endoscopy group, P = 0.01, OR = 0.149, 95% CI = 0.033-0.681) were found to be independent variables that predicted survival (Table 3).

Table 3 Multivariate logistic regression analysis for 28-d survival following first variceal hemorrhage

Variable	*P* value	OR	95% CI
MELD	0.01	1.155	1.041-1.281
Antibiotics within 8 h (peri-endoscopy)	0.01	0.149	0.033-0.681

MELD: Model for end-stage liver disease; OR: Odds ratio; CI: Confidence interval.

DISCUSSION

Acute gastrointestinal hemorrhage is associated with significant mortality, particularly when comparing variceal to non-variceal hemorrhage[31]. Historical data have reported a mortality of up to 61% following variceal hemorrhage[1-5], although current 4-6-wk mortality rates range from 14% to 20%[9-13]. Despite the advances in vasoactive drugs and endoscopic therapies, a significant contribution to the observed fall in mortality has occurred with the introduction of prophylactic broad-spectrum antibiotic therapy[23]. It has now become standard practice to administer prophylactic antibiotics in acute variceal hemorrhage and in cirrhotic patients with gastrointestinal bleeding of any cause[24,25,31].

The clear survival benefit associated with prophylactic antibiotics in gastrointestinal hemorrhage associated with cirrhosis is not in doubt. Both American and British guidelines recommend the administration of antibiotics prior to endoscopy. While this seems logical, the timings of antibiotic administration in randomized studies are heterogeneous with prophylaxis occurring before and after endoscopy, thus the exact timings are unclear[23].

The term preemptive has been used in the field of gastrointestinal hemorrhage to describe antibiotics given after an event likely to cause bacteremia (whether due to the initial bleed or subsequent endoscopy) but before clinical evidence of sepsis. We hypothesize that there is a window of opportunity for the administration of antibiotics, after which their benefit is diminished or lost. Windows of opportunity for effective use of antibiotics have been observed for other infections and patient groups. Significantly higher survival is associated with administering antibiotics within 8 h of admission for patients admitted with pneumonia[32] and within 6 h for meningitis[33]. In a mouse model of intraperitoneal infection that produced septic shock, survival was > 80% if antibiotics were given within 12 h of insult, but < 15% if given after 15 h[34]. For patients treated for septic shock in an intensive care environment, a sequential decrease in survival was noted for each hour of delay in administering antibiotics for the first 6 h[35].

The potential benefit of antibiotics in cirrhosis is balanced by the risk of adverse effects. The use of broad-spectrum antibiotics is a contributing factor to health-care-associated CDI. A recent study has shown that patients with cirrhosis who are given antibiotics are at a higher risk of developing CDI, and this is associated with a higher mortality compared to cirrhotic patients without CDI[30].

The rates of CDI within the cirrhotic patients in our study were no higher than in non-cirrhotic, general medical patients who receive a minimum 5-d course of broad-spectrum antibiotics for non-gastrointestinal indications. Both groups of patients were treated in the same ward areas and were matched for environmental risks and isolation procedures that are known to affect rates of CDI[28]. Cirrhotic patients were younger than general medical patients. However, elderly medical patients over 80 years of age and at highest risk of CDI were excluded.

This study has shown that, if given before, during or within 8 h of the index endoscopy, peri-endoscopy antibiotics are associated with a significantly improved survival at 28 d, and show a trend towards reducing 28-d rebleeding rate. The present study did not address the precise time for peri-endoscopy antibiotics, which needs to be addressed by prospective studies. This highlights the requirement for care pathways to be modified, in this unit and similar units, if the current guidelines for antibiotic administration before endoscopy are to be widely adopted.

The administration of antibiotics within 8 h importantly reduces rates of mortality and rebleeding independently of liver disease severity, judged by MELD and Child-Pugh score, in agreement with previous studies[8,11,12]. The proportion of patients who received prophylactic antibiotics in our study (91.4%) is comparable to other recently published data[12]. Similarly, the 28-d survival for first variceal hemorrhage (81.4%) is comparable to that in the current literature for all variceal bleeding (80%-84%)[9-12], and reflects the changes in standard care in the treatment of variceal hemorrhage over the past 30 years[12].

Patients with second or subsequent variceal hemorrhage were excluded from this study. Previous hospital admission for variceal hemorrhage, particularly if the admission was recent, would have been exposed to broad-spectrum antibiotics and nosocomial infection, including *C. difficile*, and potentially created heterogeneous groups. Similarly, patients transferred to the liver unit from another institution were likely to have been exposed to antibiotics prior to their transfer, and were excluded for similar reasons.

There are several strengths to this study. All included cases were first variceal hemorrhages. Medical notes were reviewed to identify hospital attendances during the 28 d prior to admission for variceal hemorrhage and involved an antibiotic prescription. Any patient who received antibiotics in this run-in period was excluded from the study. All patients were managed in a tertiary referral center and received the standard medical management for variceal hemorrhage. The medical records, clinical outcome data and rates of CDI were available for all included cases, which allowed accurate assessment of the timing of antibiotics. The use of the microbiology database ensured all cases of CDI that occurred after discharge from hospital would be included.

It is acknowledged that this is a retrospective study rather than a prospective trial, which has inherent limitations. To minimize the effects of confounding factors, we attempted to study a homogenized population, managed by standard protocols in the same environment. Cases of *C. difficile* toxin-positive stools were not assessed for severity, either clinically or endoscopically. However, all cases of CDI were in patients alive at 28 d and CDI did not predict rebleeding or death. All efforts were made to exclude patients who had received antibiotics within 28 d of admission. However, any antibiotic prescription made by a primary care physician during this period would have been unknown to the authors, unless declared by the patient. Finally, the study did not include cases of non-variceal hemorrhage in patients with cirrhosis, therefore, the findings cannot be extended to this group.

In conclusion, following variceal hemorrhage, the use of peri-endoscopy antibiotics (administered before, during or up to 8 h after endoscopy) is associated with a significant increase in survival and a trend towards a reduction in rebleeding at 28 d. Peri-endoscopy antibiotic administration is an independent variable for survival in addition to the severity of liver disease. Despite growing concerns about rising healthcare-associated infections and the use of broad-spectrum antimicrobials in cirrhosis, the rates of CDI are comparable to other patient groups who receive broad-spectrum antibiotic therapy, and should not be a reason to withhold antibiotics.

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COMMENTS

Background

Cirrhosis is a common and serious cause of intestinal hemorrhage. Twenty-two

to sixty-one percent of patients with cirrhosis during follow-up studies develop variceal bleeding, usually within 2 years of diagnosis. Variceal hemorrhage is associated with a mortality of 14%-20%. In addition to advances in endoscopic and pharmacological therapies, the prophylactic use of antibiotic drugs has reduced mortality by 27% and infection, the leading cause of death in these circumstances, by 60%. It is unclear if there is a time point after which the benefit of antibiotics is lost.

Research frontiers

Several studied have investigated the use of prophylactic antibiotic therapy following variceal hemorrhage in patients with cirrhosis after the recognition that infection leading to sepsis was a leading cause of death in these patients. Eleven key studies were included in a Cochrane meta-analysis which concluded that antibiotic treatment reduced mortality by 27% and infection by 60%. It is now routine practice to administer antibiotics following variceal bleeding; the timing of administration is unclear in the literature. The increasing use of broad-spectrum antibiotics is associated with serious side effects and complications including *Clostridium difficile* infection (CDI). Patients with cirrhosis are at increased risk of infection due to suppression of the immune system as a direct result of chronic liver disease. It is currently unknown if patients with cirrhosis receiving antibiotics following variceal hemorrhage are at increased risk for acquiring CDI.

Innovations and breakthroughs

Emergency endoscopy is an important technique for the treatment of active variceal bleeding and also for preventing subsequent future episodes of rebleeding. Endoscopic techniques such as variceal band ligation (in which rubber bands are positioned around the bleeding varix) have been shown to be more effective than older therapies such as ethanolamine injection sclerotherapy, and have become the method of choice in these situations. Similarly, vasoactive drug therapies, particularly terlipressin, have been shown to participate in the cessation of bleeding and reduce mortality by redirecting blood flow away from the splanchnic circulation. Transjugular intrahepatic portosystemic shunting is an invasive procedure performed by an interventional radiologist to reduce the portal venous pressure and arrest acute bleeding by placing a stented shunt through the liver, thus connecting the portal and hepatic veins directly. It is currently used in patients with persistent or recurrent variceal bleeding despite maximal endoscopic and pharmacological intervention. It is not currently widely available and is a second-line treatment option in complicated and persistent variceal hemorrhage.

Applications

The article shows that early antibiotics given within 8 h of endoscopy are associated with reduced mortality. Early administration of antibiotics in this situation should be adopted by units that treat patients with acute variceal bleeding and may need to be incorporated into clinical care pathways in acute admission and acute gastrointestinal bleeding units. Further prospective studies might be necessary to clarify the optimal timing of antibiotic administration following variceal hemorrhage. Patients are at no greater risk of CDI than non-cirrhotic patients who are receiving antibiotics, and concerns regarding healthcare-associated infections should not deter clinicians from prescribing antibiotic therapy in this situation.

Peer review

This is a well-written paper that is limited in impact by observational design and single-center patient population.

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BRIEF ARTICLE

Endoscopic mucosal resection of colorectal polyps in typical UK hospitals

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Abstract

AIM: To evaluate the outcomes of endoscopic mucosal resection (EMR) for colorectal polyps, with particular regard to procedural complications and recurrence rate, in typical United Kingdom (UK) hospitals that perform an average of about 25 colonic EMRs per year.

METHODS: A total of 239 colorectal polyps (≥ 10 mm) resected from 199 patients referred to Rochdale Infirmary, Salford Royal Hospital and Royal Oldham Hospital for EMR between January 2003 and January 2009 were studied.

RESULTS: The mean size of polyps resected was 19.6 ± 12.4 mm (range 10-80 mm). The overall major complication rate was 2.1%. Complications were less frequent with non-adenomas compared with the other groups (Pearson's χ^2 test, $P < 0.0001$). Resections of larger-sized polyps were more likely to result in complications (unpaired *t*-test, $P = 0.021$). Recurrence was associated with histology, with carcinoma-*in-situ* more likely to recur compared with low-grade dysplasia [hazard ratio (HR) 186.7, 95% confidence interval (95% CI): 8.81-3953.02, $P = 0.001$]. Distal lesions were also more likely to recur compared with right-sided and transverse colon lesions (HR 5.93, 95% CI: 1.35-26.18, $P = 0.019$).

CONCLUSION: EMR for colorectal polyps can be performed safely and effectively in typical UK hospitals. Stricter follow-up is required for histologically advanced lesions due to increased recurrence risk.

Key words: Endoscopic mucosal resection; Polyps; Endoscopic; Resection

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INTRODUCTION

The use of endoscopic mucosal resection (EMR), pio-

neered in Japan for the treatment of early gastric cancer[1], has expanded to include therapy of other early gastrointestinal malignancies and pre-cancerous lesions such as adenomas[2] or early stage colon cancers[3,4].

EMR allows the removal of tissue to the level of the muscularis propria and is a good alternative to surgery, thereby eliminating the need for surgical intervention and its associated morbidity and mortality. Early stage colon cancer, adenomas and rectal carcinoid have all been successfully removed by EMR. Resection of polyps can be performed *en bloc* or piecemeal according to the size and location of the lesion. The complication rate associated with EMR is low[5]. The most frequent adverse event is bleeding[6-10] followed by perforation[3,11,12]. Another risk following EMR is recurrence[8-10,13].

The use of EMR for colorectal polyps has become increasing popular in Western countries and has been found to be a safe and efficient treatment. However, most reports come from large, tertiary referral practices and the applicability to more typical hospitals in the UK has not been established.

This study aims to evaluate the outcomes of EMR for colorectal polyps with particular regard to procedural complications and recurrence rate across three hospitals in North West England which perform an average of about 25 colonic EMR per year.

MATERIALS AND METHODS

Study population

All colorectal polyps referred to Rochdale Infirmary, Salford Royal Hospital and Royal Oldham Hospital for EMR between January 2003 and January 2009 were considered for the study. There is one endoscopist performing the EMR procedures in each center. Polyps ≥ 10 mm in diameter were selected for the study. Non-lifting lesions or laterally spreading lesions were excluded as these were referred for open surgical resection. Polyps were identified using a detailed record of procedures performed in the endoscopy unit and operating theatres in the three hospitals both for in-patients and out-patients. A total of 239 polyps resected (from 199 patients) were identified for the study.

Resection method

EMR was performed with a standard polypectomy snare (Olympus SnareMaster SD-230U). The lesion was first lifted by injection of a large volume (10-100 mL) of a pre-mixed solution around the lesion. This consisted of 40 mL Volplex (succinylated gelatine 20 g in 500 mL), 2 mL of 1:10 000 adrenaline, and 2 mL of 0.4% indigo carmine. The open snare was placed around the lesion and was gently pressed against the mucosa. Excess air was aspirated from the colon to decrease distension and facilitate grasping of the targeted polyp. The aim was to resect lesions in one single piece rather than piecemeal if possible. After snare excision, air was insufflated to visualize the area of resection and, if needed, any further residual tissue was removed in similar fashion. Adjuvant argon plasma coagulation (APC) therapy was used to remove tiny remnants of lesions visible after resection. The settings used were "auto-cut" at a fixed power of 120 W, as recommended by the manufacturer, along with the "endo-cut" mode. APC was applied in short bursts to avoid thermal damage to the muscularis propria.

Procedures were performed with the patient under conscious sedation (intravenously administered midazolam and/or pethidine/fentanyl). When required, hyoscine-butyl bromide was given intravenously.

Assessment of dimension of polyps and histopathology

The size of polyps was estimated by comparison with open biopsy forceps and when possible after retrieval. All removed tissue was retrieved using a Roth net, retrieval basket, grasper or through the suction channel.

All retrieved polyps were examined and classified by experienced pathologists at the respective histopathology departments of each hospital. Polyps were classified as adenoma, carcinoma-*in-situ* (CIS) or non-adenoma (e.g. hyperplastic polyps, lipoma).

Adenomas were further classified according to the Konishi-Morson system for grade of dysplasia (i.e. low-, moderate-, or high-grade) using a combination of variables, including tubule configuration, nuclear polarity, orientation and structure, mucin content and location, *etc*[14]. The diagnosis of CIS included adenomas with neoplastic cells invading into the lamina propria mucosae[15,16].

Record of complications

Patients were instructed to report symptoms of continuing abdominal pain or bleeding per rectum. The diagnosis of post-procedure bleeding was based on the passage of fresh blood per rectum. Intra-procedural perforation was diagnosed by endoscopy during the resection and confirmed by air on plain abdominal film and/or abdominal computed tomography scan. Post-procedural perforation characterized by abdominal pain and leucocytosis was diagnosed by the presence of free air on plain abdominal film and/or abdominal computed tomography scan.

Follow-up and recurrence

Surveillance colonoscopy was performed at various intervals, at the discretion of the clinician in charge of the individual patient's care. During endoscopic follow-up, any alterations of the mucosa in the area of previous resection (ulcerations, scarring, retractions of mucosa, *etc*.) were biopsied. Recurrence was defined as the presence of adenomatous or polypoid tissue on a follow-up endoscopy. This was treated with a repeat EMR if possible.

Statistical analysis

Data are presented as mean ± SD and range unless otherwise stated.

Association of complications with different variables was assessed using unpaired *t*-test or Pearson's χ^2 test. In patients who underwent endoscopic surveillance, the Cox univariate analysis was used to identify significant prognostic factors. *P* values < 0.05 were regarded as statistically

Table 1 Patient demographics *n* (%)

	No. of patients (*n* = 199)
Age (yr)[1]	68.0 ± 11.4
Sex	
M	119 (59.8)
F	80 (40.2)
Polyp location	
Cecum	49 (20.5)
Ascending colon	30 (12.6)
Transverse colon	26 (10.9)
Descending colon	34 (14.2)
Sigmoid colon	43 (18.0)
Rectum	57 (23.8)
Total	239
Polyp size (mm)[1]	68.0 ± 11.4
Range (mm)	10-80

[1]Values are mean ± SD. Values are number of patients with percentages in parentheses unless indicated otherwise.

Table 2 Characteristics of polyps according to histopathology *n* (%)

	Non-adenomas	Adenomas	Carcinoma-*in-situ*
Age (yr)[1]	61.3 ± 12.9	69.2 ± 9.9	76.2 ± 11.2
Sex			
M	15	117	9
F	23	64	4
Polyp size (mm)[1]	13.9 ± 5.2	20.8 ± 13.1	22.7 ± 14.9
Polyp location			
Cecum	13 (34.2)	31 (17.1)	2 (15.4)
Ascending colon	5 (13.2)	24 (13.3)	0
Transverse colon	9 (23.7)	15 (8.3)	1 (7.7)
Descending colon	4 (10.5)	29 (16.0)	0
Sigmoid colon	6 (15.8)	33 (18.2)	3 (2.3)
Rectum	1 (2.6)	49 (27.1)	7 (5.4)
Total	38	181	13
Resection method			
En bloc	33 (86.8)	129 (71.3)	9 (69.2)
Piecemeal	5 (13.2)	50 (27.6)	4 (30.8)
Not recorded	0	2 (1.1)	0
Complete resection			
Yes	36 (94.7)	154 (85.1)	10 (76.9)
No	2 (5.3)	26 (14.4)	3 (23.1)
Not recorded	0	1 (0.5)	0
Recurrence[2]			
No		75 (48.8)	1 (10.0)
Yes		30 (19.4)	3 (30.0)
No follow up		49 (31.8)	6 (60.0)

[1]Values are mean ± SD; [2]Only completely resected lesions were assessed for recurrence. Values are number of patients with percentages in parentheses unless indicated otherwise.

significant. Statistical analysis was performed using SPSS 15.0 statistical package (SPSS Inc., Chicago, IL, USA).

RESULTS

A total of 239 polyps were treated in 199 patients. Thirty-three patients had more than one polyp. The characteristics of the patient population, and polyp size and location, are presented in Table 1. The mean age of the patients was 68.0 ± 11.4 years and approximately 60% of the patients were male. The mean size of polyps resected was 19.6 ± 12.4 mm (range 10-80 mm).

A breakdown of polyps resected according to histopathology is shown in Table 2. Of the adenomas resected, 47 (26.0%) polyps were classified as low-grade dysplasia (LGD), 77 (42.5%) moderate-grade dysplasia (MGD), 49 (27.1%) high-grade dysplasia (HGD), and the classifications of eight polyps were unrecorded.

Complete resection was achieved in 86.2%. Patients who failed to have polyps completely resected were either referred for surgery or had their polyps resected in a repeat session. These patients were excluded from surveillance analysis (see below).

Table 3 Hazard ratio for risk of recurrence associated with clinical variables (*n* = 164)

	Hazard ratio	95% CI	*P*[1]
Age	1.036	0.982-1.093	0.194
Sex	0.851	0.301-2.403	0.761
Size	1.023	0.974-1.074	0.357
Resection method (*en bloc vs* piecemeal)	0.680	0.188-2.452	0.555
Histology			0.004
MGD *vs* LGD	2.837	0.358-22.499	0.324
HGD *vs* LGD	2.009	0.206-19.630	0.549
CIS *vs* LGD	186.651	8.813-3953.020	0.001
Site (distal colon *vs* right and transverse colon)	5.933	1.345-26.175	0.019

[1]Cox univariate analysis. LGD: Low-grade dysplasia; MGD: Moderate-grade dysplasia; HGD: High-grade dysplasia; CIS: Carcinoma-*in-situ*.

Complications

Two patients had significant post-procedural bleeding. One patient responded to intravenous fluid resuscitation and blood transfusion, while the other required a sigmoid colectomy to treat the bleeding. Two patients developed post-procedural perforation. One patient was managed conservatively; while the other went on to have surgery (anterior resection of rectum). No procedure-related mortality was reported. The overall major complication rate was 2.1%.

Complications were less frequent with non-adenomas compared with the other groups (Pearson's χ^2 test, $P < 0.0001$). Resections of larger-sized polyps were more likely to result in complications (unpaired *t*-test, $P = 0.021$).

Surveillance

Seventy-eight out of 139 patients (164 polyps) with completely resected adenomas and CIS underwent follow-up colonoscopy (56.1%). Median follow up was 6.8 mo (range 1.2-26.6 mo).

Local recurrence was detected at 33 resection sites. The histopathology of the recurrent polyps was as follows: 7 (21.2%) LGD, 16 (48.5%) MGD, 7 (21.2%) HGD, and 3 (9.1%) CIS.

On univariate analysis (Table 3), recurrence was associated with histology, with CIS more likely to recur compared with LGD [hazard ratio (HR) 186.7, 95% con-

fidence interval (95% CI): 8.81-3953.02, $P = 0.001$]. Distal lesions were also more likely to recur compared with right-sided and transverse colon lesions (HR 5.93, 95% CI: 1.35-26.18, $P = 0.019$).

DISCUSSION

Several studies have been published regarding the safety and therapeutic potential of endoscopic resection of colorectal polyps. The most frequent adverse event of EMR reported is bleeding, occurring in 1% to 45% of cases[6-10]. Perforation rate has been reported to be between 0.7% and 4%[3,11,12]. The overall serious complication rate in the present study is only 2.1%, although one of the main drawbacks of the study is the lack of data on intra-procedural bleeding. However, this is often self-limiting or can be treated successfully during the procedure. Complication rates of EMR are lower than those of open surgery (2.3% to 6.3%) and comparable to those of laparoscopic surgery (1.9% to 6.9%)[17]. However, mean hospital stay for the patients who undergo colorectal surgery is 9.2-13.2 d, significantly longer than for patients who undergo EMR procedures, which are usually performed as day cases[17]. Therefore, EMR is a good alternative to surgery in a selected group of patients unfit for surgery.

The present study highlights that increasing size of polyp resected is associated with complications and the authors recommend that extra care should be taken in resecting polyps of > 35 mm in diameter.

Where possible all lesions were removed in a single session as this eliminates the discomfort and the inconvenience of repeated procedures for patients. This was achieved in 86.2% of resections in the present study. Preference has been suggested in some studies for *en bloc* resection compared with piecemeal resection, because it provides more accurate histological assessment and reduces the risk of local recurrence[18]. However, in the present study there was no statistically significant difference in the recurrence rate reported between the two resection techniques.

Follow-up is essential because of the risk of recurrence. Aggressive surveillance seems justified because it has been shown, in an animal model, that residual tumor has a high regrowth rate[19]. Unfortunately, only about half of the patients in the present study underwent a follow-up colonoscopy. The main reasons for non follow-up were patient refusal and patient frailty.

Recurrence rates after EMR of colorectal polyps have been reported to be between 0% and 46%[8-10,13]. In the present study, the recurrence rates were 19.5% for non-malignant polyps and 30% for malignant polyps. Several studies have suggested that size of polyps is associated with recurrence. However, this was not found in the present study. Instead, recurrence was more common in distal and/or more histologically advanced lesions.

The present study has shown that EMR for colorectal polyps can be carried out safely and effectively in typical UK hospitals provided the endoscopists carrying out the procedures have the appropriate training and interest in EMR. This procedure should be considered in preference to open surgery, especially in polyps smaller than 35 mm in diameter. Also, a stricter follow-up may be required for more histologically advanced lesions because of a higher risk of recurrence.

COMMENTS

Background

The use of endoscopic mucosal resection has expanded to include therapy of early gastrointestinal malignancies and pre-cancerous lesions such as adenomas or early stage colon cancers, especially in Western countries. However, most studies have come from large tertiary centers in the world.

Research frontiers

In this study, the authors demonstrate that endoscopic mucosal resection of colorectal polyps can be performed safely and effectively in typical United Kingdom (UK) hospitals, with complication rates and recurrence rates which are comparable to tertiary centers.

Innovations and breakthroughs

This is the first study to report results from the practice of endoscopic mucosal resection of colorectal polyps in typical UK hospitals where an endoscopist performs an average of 25 of these procedures per year.

Applications

It can be established from this study that patients from non-tertiary centres which have similar experience in endoscopic mucosal resection may be reassured that the procedures can be performed safely and effectively.

Terminology

Endoscopic mucosal resection allows the removal of tissue to the level of the muscularis propria and is a good alternative to surgery, thereby eliminating the need for surgical intervention and its associated morbidity and mortality.

Peer review

The take home message from this study is that it is feasible to perform endoscopic mucosal resection in a community hospital with a trained endoscopist.

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BRIEF ARTICLE

Factors associated with incomplete small bowel capsule endoscopy studies

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Abstract

AIM: To identify patient risk factors associated with incomplete small bowel capsule endoscopy (CE) studies.

METHODS: Data from all CE procedures performed at St. Paul's Hospital in Vancouver, British Columbia, Canada, between December 2001 and June 2008 were collected and analyzed on a retrospective basis. Data collection for complete and incomplete CE study groups included patient demographics as well as a number of potential risk factors for incomplete CE including indication for the procedure, hospitalization, diabetes mellitus with or without end organ damage, limitations in mobility, renal insufficiency, past history of bowel obstruction, abdominal surgery, abdominal radiation therapy and opiate use. Risk factors were analyzed using a univariable and multivariable logistic regression model.

RESULTS: From a total of 535 CE procedures performed, 158 were incomplete (29.5%). The univariable analysis showed that CE procedures performed for overt gastrointestinal bleeding (P = 0.002), and for patients with a prior history of abdominal surgery (P = 0.023) or bowel obstruction (P = 0.023) were significantly associated with incomplete CE studies. Patients on opiate medications (P = 0.094) as well as hospitalized patients (P = 0.054) were not statistically significant, but did show a trend towards incomplete CE. The multivariable analysis showed that independent risk factors for an incomplete CE procedure include prior history of bowel obstruction [odds ratios (OR) 2.77, P = 0.02, 95% confidence intervals (CI): 1.17-6.56] and procedures performed for gastrointestinal bleeding (Occult OR 2.04, P = 0.037, 95% CI: 1.04-4.02 and Overt OR 2.69, P = 0.002, 95% CI: 1.44-5.05). Patients with a prior history of abdominal surgery (OR 1.46, P = 0.068, 95% CI: 0.97-2.19), those taking opiate medications (OR 1.54, P = 0.15, 95% CI: 0.86-2.76) and hospitalized patients (OR 1.82, P = 0.124, 95% CI: 0.85-3.93) showed a trend towards statistical significance.

CONCLUSION: We have identified a number of risk factors for incomplete CE procedures that can be used to risk-stratify patients and guide interventions to improve completion rates.

Key words: Capsule; Capsule endoscopy; Incomplete endoscopy

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INTRODUCTION

Capsule endoscopy (CE) is a novel and non-invasive imaging modality for the small bowel initially described in 2000[1,2]. The procedure utilizes a battery-powered wireless capsule to transmit images of the gastrointestinal tract as it traverses through the intestine. These transmitted images are sent to a recorder worn on the abdominal wall of the patient. The images are later downloaded to a computer and interpreted by a trained physician.

There are a number of indications for the use of CE in small bowel diseases. One of the most common indications for a CE study is to investigate obscure gastrointestinal bleeding, but other indications include inflammatory bowel disease, small bowel tumors, familial polyposis syndromes and celiac disease[1,3-8]. Various studies have shown favorable results in using CE in the aforementioned small bowel diseases[3-10]. Furthermore, studies have also shown that CE has better diagnostic yield when compared to other modalities of imaging the small bowel such as push-enteroscopy or computed tomography angiography in the diagnosis of obscure gastrointestinal bleeding[9,10].

However, it is important to note that CE is not without limitations. There are, albeit rare, reports of capsule retention in the gastrointestinal tract as well as aspiration of capsule devices[11,12]. Furthermore, a retrospective study carried out in 2005 revealed a technical limitation/failure rate of 8.5% which was due to factors such as downloading or transmitting failures, insufficient battery life, or signal interference of the devices[13]. A relatively common limitation is the issue of incomplete CE studies, which can be due to the capsule not reaching the cecum and/or poor visualization of a significant portion of the small bowel due to factors such as poor preparation or excess debris. A number of studies have shown rates of incomplete CE procedures to be greater than 13%[13-15].

CE studies that are incomplete or difficult to interpret due to poor visualization potentially lead to delays in the diagnosis of small bowel pathology. Further investigative tests such as repeat CE studies, radiologic exams, or endoscopic interventions are often required to help delineate the diagnosis. Ultimately, there are increased costs to the medical system and inconvenience to the patient with delays in diagnosis.

The aim of this study is to determine demographic and procedure process related risk factors associated with incomplete CE studies. A number of small studies have identified factors such as diabetes mellitus, hospitalization, slowed gastric transit, previous small bowel surgery, and poor bowel cleansing[14,16,17]. Data for this study are drawn from the capsule endoscopies performed at St. Paul's Hospital, a quaternary referral center for gastroenterology services and the only referral center for CE in the province of British Columbia, Canada.

MATERIALS AND METHODS

CE procedure

All CE procedures were performed at St. Paul's Hospital in Vancouver, British Columbia, Canada. Prior to the procedure, informed consent was obtained and all patients were given a standardized set of instructions. The instructions included an overnight fast beginning at midnight before the procedure, as well as a bowel-cleansing regimen consisting of a single bottle (300 mL) of Magnesium citrate. Motility agents were not routinely used during the procedure; however, if patients were already taking these medications, they were permitted to continue taking them. All CE procedures began at 7 am and were completed by 3 pm on the same day, at which time the data recorders would be returned. The images were subsequently downloaded to a computer and reviewed within 24 h by a single gastroenterologist (RE). Patients were permitted to drink within 2 h and eat within 4 h following ingestion of the capsule. All CE procedures were performed using the PillCam produced by Given Imaging (Yoqneam, Israel). Incomplete CE studies were defined as those that did not reach the cecum or had incomplete visualization of the small bowel due to debris or incomplete cleansing (involving > 25% of the study). We included incomplete visualization of the small bowel to our definition of an incomplete CE study to provide an estimate of incomplete capsules in a real-world clinical setting.

Data collection

Data from all CE studies performed between December 2001 and June 2008 were reviewed and analyzed on a retrospective basis. An incomplete CE study was defined as: (1) the capsule did not reach the cecum by the end of battery life; and (2) poor visualization of > 25% of the mucosa. To determine potential risk factors for incomplete CE studies using univariable and multivariable statistical analysis, data on a number of potential factors were collected: (1) The indication for the CE (iron deficiency, abdominal pain, change in bowel habits, active GI bleed, occult GI bleeding, recurrent GI bleed); (2) Whether the study was performed on a hospitalized patient; (3) Diabetes mellitus with or without end organ damage (defined as diabetic nephropathy, retinopathy, neuropathy or diabetes-associated peripheral vascular disease); (4) Limitations in mobility (i.e. post stroke, hemiplegia), (5) Renal insufficiency; (6) Patient demographics (age, gender); (7) Past history of abdominal surgery, bowel obstruction, or abdominal radiation therapy; and (8) Opiate use.

Statistical analysis

All statistical analyses were performed using STATA 10.0 (College Station, TX). Descriptive statistics were used to characterize the demographics of the patient population. Patients with incomplete studies were compared to those with completed studies and risk factors for an incomplete CE were compared using a univariable and then multivariable logistic regression. Hemiplegia and diabetes with end organ damage were risk factors that occurred in a low

Table 1 Patient demographics (%)

	Complete CE	Incomplete CE	Total
n	377	158	535
Gender (F)	50.9	50.0	50.7
Age (yr)	58.1 (13.2-92.9)	60.6 (12.8-90.7)	58.8 (12.8-92.9)
Hospitalized	4.5	8.8	5.8
Diabetes	12.7	16.5	13.8
Hemiplegia	0.27	1.3	0.56
Renal disease	6.6	8.2	7.1
DM with EOD	2.7	2.5	2.6
No bleed	22.3	18.4	19.3
Occult bleed	30.9	26.0	30.0
Overt bleed	46.8	55.6	50.7
Change in BM	17.5	17.7	17.6
Bowel obstruction	3.4	8.2	4.9
Abdominal surgery	34.5	44.9	37.6
Abdominal radiation	1.9	1.9	1.9
Opiate use	8.8	14.8	10.7

CE: Capsule endoscopy; DM: Diabetes mellitus; EOD: End organ damage; BM: Bowel movement.

Table 2 Univariable analysis

	B coefficient	*P* value	OR	95% CI	Pseudo R-squared
Gender (F)	-0.0371	0.845	0.96	0.66-1.39	0.0001
Age	0.00759	0.152	1.01	0.99-1.02	0.0032
Hospitalized	0.722	0.054	2.06	0.99-4.29	0.0055
Diabetes	0.3	0.256	1.35	0.80-2.27	0.0019
Hemiplegia	1.57	0.200	4.82	0.43-53.5	0.0027
Renal disease	0.233	0.513	1.26	0.63-2.54	0.0007
DM with EOD	-0.048	0.936	0.95	0.29-3.09	0.00001
No bleed	Reference				0.0167
Occult bleed	0.517	0.095	1.68	0.91-3.07	
Overt bleed	0.87	0.002	2.39	1.36-4.16	
Change in BM	0.0148	0.952	1.01	0.62-1.65	0.00001
Bowel obstruction	0.92	0.023	2.51	1.13-5.55	0.0078
Abdominal surgery	0.439	0.023	1.55	1.06-2.26	0.0079
Abdominal radiation	0.0228	0.974	1.02	0.26-4.00	0.00001
Opiate use	0.479	0.094	1.61	0.92-2.82	0.0042

DM: Diabetes mellitus; EOD: End organ damage; BM: Bowel movement; OR: Odds ratio; CI: Confidence interval.

number of patients and were therefore excluded. From the multivariable regression analysis, odds ratios (OR) and corresponding 95% confidence intervals (CI) were generated on all significant variables (*P*-value < 0.05). Patients excluded from statistical analysis include those with evidence of mass lesion or stricture that was responsible for delaying transit through the small bowel.

RESULTS

A total of 653 CE procedures were reviewed in total. However, 118 studies were excluded, as there was evidence of a mass lesion or stricture that clearly delayed transit through the small bowel and was felt to be primarily responsible for the incomplete study. Therefore, statistical analysis was performed on a total of 535 CE procedures. Of those 535 CE procedures, 158 CE were found to be incomplete (29.5%).

Patient demographics

Patient demographic information for complete and incomplete CE studies is seen in Table 1. The mean age of the patients was 58.8 years, and 50.9% of patients were female. There was no significant difference in the demographics as well as the indication for performing the CE procedure between complete and incomplete CE study groups.

Univariable analysis

Results of the univariable analysis are listed in Table 2 for all investigated potential risk factors for incomplete CE studies listed in Table 1. The analysis reveals that factors strongly associated with incomplete CE studies include overt GI bleeding ($P = 0.002$, OR 2.39, 95% CI: 1.36-4.16), as well as prior history of bowel surgery ($P = 0.023$, OR 1.55, 95% CI: 1.06-2.26) and bowel obstruction ($P = 0.023$, OR 2.51, 95% CI: 1.13-5.55). Patients on opiate medications ($P = 0.094$, OR 1.61, 95% CI: 0.92-2.82) as well as those admitted to hospital ($P = 0.054$, OR 2.06, 95% CI: 0.99-4.29) were not statistically significant risk factors, but clearly showed a trend towards significance.

Table 3 Multivariable analysis

	OR	*P* value	95% CI
Sex	0.99	0.975	0.68-1.46
Age	0.99	0.995	0.99-1.01
Hospitalized	1.82	0.124	0.85-3.93
Diabetes	1.25	0.411	0.73-2.17
Renal disease	1.03	0.926	0.50-2.16
Occult bleeding	2.04	0.037	1.04-4.02
Overt bleeding	2.69	0.002	1.44-5.05
PMHx bowel obstruction	2.77	0.020	1.17-6.56
PMHx abdo surgery	1.46	0.068	0.97-2.19
PMHx abdo radiation	0.91	0.890	0.22-3.71
Opiate use	1.54	0.150	0.86-2.76

PMHx: Past medical history; OR: Odds ratio; CI: Confidence interval.

Multivariable analysis

Results of the multivariate analysis, shown in Table 3, using significant risk factors determined from the univariable analysis, show a statistically significant independent risk of incomplete CE study in patients who completed the study for a history of overt (OR 2.69, $P = 0.002$, 95% CI: 1.44-5.05) or occult (OR 2.04, $P = 0.037$, 95% CI: 1.04-4.02) gastrointestinal bleeding as well as patients with a previous history of bowel obstruction (OR 2.77, $P = 0.02$, 95% CI: 1.17-6.56). A prior history of abdominal surgery (OR 1.46, $P = 0.068$, 95% CI: 0.97-2.19) lacked statistical significance, but did show a trend towards an incomplete CE study. Patients on opiate medications (OR 1.54, $P = 0.15$, 95% CI: 0.86-2.76, 130/377 in the complete group, 71/158 in incomplete group) and those who were hospitalized (OR 1.82, $P = 0.124$, 95% CI: 0.85-3.93, 33/377 in the complete group, 23/158 in the incomplete group) also showed a trend towards statistical significance.

DISCUSSION

Our study intended to determine risk factors associated with incomplete small bowel CE studies due to poor visualization or failure of the capsule to reach the cecum. In order to accomplish this task, we have reviewed all CE studies performed at our center over a seven-year period. We identified 158 incomplete studies from a total of 535 eligible capsules (29.5%), showing that our incomplete CE study rate is higher than reports from other papers[13-15]. This is likely because we were more inclusive in the definition of an incomplete CE study compared to previous studies, as we have included poor visualization of > 25% of the mucosa into the definition rather than only counting capsules that did not reach the cecum[13-15]. We added both aforementioned scenarios into our definition to provide a real-world clinical estimate of the rate of incomplete capsule endoscopies.

A number of prior studies have already identified risk factors associated with incomplete CE studies such as hospitalization, diabetes mellitus, prolonged gastric transit times, and poor bowel cleansing[14,16,17]. Our results have largely added to this list of established risk factors. The result of our univariable analysis showed that factors strongly associated with incomplete studies include a past history of bowel obstruction or abdominal surgery, as well as studies done for overt gastrointestinal bleeding. Hospitalized patients and those on opiates showed a trend towards significance for incomplete CE studies. Furthermore, the multivariable analysis revealed that a prior history of bowel obstruction and studies performed for gastrointestinal bleeding (both overt and occult) were independent risk factors for incomplete studies, with a past history of abdominal surgery, hospitalization and opiate use showing a trend towards significance.

Given that there are a number of studies that have investigated risk factors for incomplete CE procedures, including a recent article published by Westerhof *et al*[14,16,17] in 2009 that, like our paper, provided a retrospective univariable and multivariable analysis of factors associated with incomplete small bowel CE procedures, we feel that our study contributes to the literature in a number of ways. Firstly, our study provides a systematic analysis of risk factors for small bowel CE procedures with the largest patient population available in the literature at the present time. Secondly, we have included a variety of potential risk factors such as hospitalization, opiate use, mobility limitations and systemic illness into our statistical model. And thirdly, we have included a functional definition of an incomplete study, being more inclusive than other studies to give a better real-world estimate of the proportion of CE studies that are incomplete or difficult to interpret.

In terms of how this data affects our practice, we have used this information to risk-stratify patients undergoing CE studies to determine if they are indeed at high risk for an incomplete study. If a patient is deemed to be at high risk, we have made changes to our practice such as utilizing the MiRo capsule (IntroMedic Co., Ltd., Seoul, Korea), a device that is capable of a longer battery life compared to other capsules, in order to continue filming the gastrointestinal tract in those with slowed intestinal motility. We also utilize a stronger bowel-cleansing regimen in those who are deemed to be at high risk for an incomplete study, which includes 4 L of Go-Lytely on the day prior to the procedure rather than a standard single bottle of Magnesium citrate.

There have been limited numbers of published studies that have investigated the use of prokinetic agents such as metoclopramide and erythromycin to improve completion rates in CE studies[17-20]. However, because of the paucity of data and heterogeneity of results seen in the literature surrounding prokinetic agents in CE, its routine use has remained controversial[20]. Because of this controversy, we do not routinely use prokinetic agents during CE studies, nor do we use them in patients deemed to be at high risk for an incomplete study. However, we feel that this would be an interesting area of research in the future to see if the routine use of prokinetic agents in a large patient population can improve completion rates.

Finally, it is important to note that our study includes a large patient population where we have retrospectively looked at numerous risk factors for incomplete CE studies. We acknowledge that there are limitations to a retrospective statistical analysis, and as a result, we feel that larger scale studies performed on a prospective basis are required to further delineate the risk factors identified. We hope that further studies can shed light on a number of issues, such as determining optimal preparations for capsule studies since there is much heterogeneity between centers, analyzing the cost-effectiveness of CE given the high incompletion rates quoted in papers, and finally, determining positive predictors of diagnostic yield in CE to aid clinicians in determining appropriate patient selection for this procedure.

COMMENTS

Background

Capsule endoscopy (CE) is a relatively new imaging modality for the small bowel initially described in 2000. It utilizes a pill-shaped camera to image the small bowel as it traverses through. CE is currently being used to diagnose and follow patients with a variety of conditions such as gastrointestinal bleeding, small bowel tumors, inflammatory bowel diseases, and celiac disease. This study focuses on determining risk factors that lead to incomplete CE procedures (i.e. capsules which fail to traverse the entire small bowel or do so but encounter so much debris that the images cannot be interpreted). This study determined that there is an incomplete rate of 29.5% in CE procedures.

Research frontiers

Future areas of research will include determining optimal preparations to be used in patients prior to undergoing a CE procedure. In addition, given the relatively high rate of incomplete procedures, studies that determine cost-effectiveness of the procedure and that determine which groups to offer the procedure to (i.e. which groups of patients will have the greatest yield by completing this procedure) will constitute future areas of research.

Innovations and breakthroughs

Prior studies have already established a number of risk factors for incomplete CE studies. These include patients with a history of diabetes mellitus, procedures performed on hospitalized patients, patients with poor bowel cleansing prior to the procedure, and those with delayed gastric emptying. The authors have added to this list of risk factors patients with a history of bowel obstruction or abdominal surgery, those taking opiate medications, hospitalized patients,

as well as studies performed for gastrointestinal bleeding. To accomplish this, they have collected data and performed statistical analysis on a large patient population at a Canadian academic teaching hospital.

Applications

Once specific risk factors can be determined for incomplete CE studies, one can use this information to provide patients with interventions to modify the risk factors (i.e. different bowel cleansing regimens, prokinetic agents, using capsules with longer battery life) in the hopes of improving CE study completion rates.

Terminology

Capsule endoscopy: A procedure that utilizes a pill-shaped camera that is swallowed by patients and is used to image the gastrointestinal tract as it traverses through. Capsules have a limited battery life and are expected to reach the end of the small bowel before time runs out. Prokinetic agent: A medication given to speed the emptying of stomach contents. Bowel cleansing regimen: A medication given to patients prior to undergoing a CE procedure to clean out the bowels such that improved visualization of the mucosa can be achieved.

Peer review

The paper is well designed and structured for both the aims and results. The discussion is too large and redundant and should be shortened.

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BRIEF ARTICLE

MK615 decreases RAGE expression and inhibits TAGE-induced proliferation in hepatocellular carcinoma cells

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Abstract

AIM: To investigate the proliferative effect of advanced glycation end-products (AGEs) and the role of their cellular receptor (RAGE) on hepatocellular carcinoma (HCC) cells, and the inhibitory effects of MK615, an extract from Japanese apricot, against AGEs were also evaluated.

METHODS: Two HCC cell lines, HuH7 and HepG2, were used. Expression of RAGE was investigated by polymerase chain reaction, Western blotting, and flow cytometry (FACS). The effect of MK615 on RAGE expression was also evaluated by FACS. The proliferative effects of a control (unglycated bovine serum albumin), glucose-derived AGEs (Glc-AGE), and glyceraldehyde-derived AGEs (Glycer-AGE), and the anti-proliferative effect of MK615 against AGEs, were evaluated using MTT assays.

RESULTS: Expression of RAGE was confirmed at both the mRNA and protein levels in both HuH7 and HepG2. FACS revealed that the level of RAGE expression was higher in HuH7 than in HepG2. Treatment with 0.1 μg/mL MK615 decreased the expression level of RAGE from 24.3% to 3.7% in HuH7 and from 6.2% to 4.8% in HepG2. The growth indices for the control, Glc-AGE, and Glycer-AGE were 1.06 ± 0.08, 0.99 ± 0.04, and 1.38 ± 0.05, respectively, in HuH7 (P = 0.037), and were 1.03 ± 0.04, 1.04 ± 0.03, and 1.07 ± 0.05, respectively, in HepG2 (P > 0.05). When the cells were cultured simultaneously with Glycer-AGE and MK615, MK615 abrogated the proliferative effect of Glycer-AGE in HuH7.

CONCLUSION: Only Glycer-AGE has a proliferative effect on HuH7, which expresses a higher level of RAGE. MK615 suppresses the proliferative effect of Glycer-AGE on HuH7 by decreasing the expression of RAGE.

Key words: Hepatocellular carcinoma; Receptor of advanced glycation end-product; Advanced glycation end-products; MK615; Toxic advanced glycation end-products

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INTRODUCTION

MK615, an extract from the Japanese apricot, *Prunus mume* Sieb. et Zucc (*ume* in Japanese), contains several triterpenoids and other unknown components. We have previously demonstrated the anti-cancer effects of MK615, which induces apoptosis[1] and autophagy[2] in cancer cells by modulation of the cell cycle through inhibition of aurora kinases A and B[3,4]. It has also been shown that MK615 exerts its anti-inflammatory effects by inhibiting the release of high mobility group box 1 protein (HMGB1) through activation of the transcription factor, Nrf2[5]. The nuclear protein HMGB1 is released into the extracellular space during necrosis and apoptosis, and causes inflammation[6]. The cellular receptor of HMGB1 is a receptor of advanced glycation end-products (AGEs), and is known as receptor of AGE (RAGE). Thus, HMGB1 and AGEs share the same cellular receptor, RAGE.

AGEs are products of non-enzymatic, irreversible glycation of proteins, and are formed under conditions of sustained hyperglycemia[7]. Binding of AGEs to RAGE initiates a potent inflammation response and induces various pathological conditions[8], such as diabetic complications[9], Alzheimer's disease[10], non-alcoholic steatohepatitis[11], and cancers[12].

Although the cellular events that operate in the AGEs/RAGE system are not fully understood, it has been shown that binding of AGEs to RAGE produces reactive oxygen species[13] and increases the transcription of vascular endothelial growth factor (VEGF), followed by angiogenesis[14]. Also, the AGEs/RAGE system activates p38/MAP kinase[15] and nuclear factor (NF)-κB[16]. These cellular events induce the production of proinflammatory cytokines[6].

Because AGEs constitute a heterozygous group, some molecules that are categorized as AGEs are not particularly toxic, including N-(carboxymethyl) lysine (CML) and pyrroline. Accumulated evidence indicates that glyceraldehyde-derived AGEs (Glycer-AGE) have a predominantly toxic structure, and these are referred to as toxic AGEs (TAGE)[10,17]. Although a number of studies have investigated the roles of various AGEs, including CML, pyrroline, and glucose-derived AGEs (Glc-AGE), and also the function of RAGE in cancers[18,19], the role of Glycer-AGE in tumorigenesis still remains unclear, especially in hepatocellular carcinoma (HCC).

Recently, it was shown that MK615 inhibits the activation of ERK1/2, p38MAPK, and NF-κB by the AGEs/RAGE system, and suppresses the release of proinflammatory cytokines[20].

The purposes of the present study were, using HCC cell lines, to determine (1) the type of effect that MK615 exerts on HCC; (2) whether Glycer-AGE has a proliferative effect on HCC; and (3) whether MK615 attenuates the effect of Glycer-AGE on HCC.

MATERIALS AND METHODS

Cells and culture

Two human hepatocellular carcinoma cell lines, HuH7 and HepG2, were used. HuH7 was purchased from the Cell Resource Center of the Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan), and HepG2 was purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS) in an incubator with a 5% CO_2 atmosphere.

Agents

AGEs: Unglycated bovine serum albumin (BSA) (control), glucose-derived AGEs (Glc-AGE:AGE1), and glyceraldehyde-derived AGEs (Glycer-AGE:AGE2) were produced by one of the co-authors, M. Takeuchi, as described previously[21,22]. Briefly, BSA at 25 mg/mL was incubated under sterile conditions with 0.5 mol/L D-glucose for 8 wk (Glc-AGE), or with 0.1 mol/L D-glyceraldehyde for 7 d (Glycer-AGE) at 37℃. Then, low-molecular-weight reactants and sugars were removed using PD-10 column (GE Healthcare UK Ltd., Buckinghamshire, England) chromatography and dialysis against phosphate-buffered saline (PBS). Control unglycated BSA was incubated under the same conditions except for an absence of reducing sugar. Preparations were tested for endotoxin using an Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan). For all experiments, the control, Glc-AGE, and Glycer-AGE were used at a concentration of 0.1 mg/mL in the culture medium.

MK615: MK615 was provided by AdaBio Co., Ltd. (Gunma, Japan). MK615 is derived from Japanese apricot fruit[1-4]. Briefly, the preparation procedure involves extraction of the apricot juice using a press, and this is then heated and concentrated. The concentrate is dissolved in water, and then adjusted to pH 7.0 with NaOH. The solution is finally sterilized in an autoclave. MK615 was used at a concentration of 0.1 μg/mL[5], except for the experiment shown in Figure 1.

Polymerase chain reaction

Cells were plated at 1×10^5/well in 24-well plates and cultured for 24 h. Then, MK615 (0.1 μg/mL) was added to the medium and culture was continued for another 24 h. Total RNA from each cell line was isolated using a Total RNA Isolation kit (MACHEREY-NAGEL, Düren, Germany). Reverse transcription reactions were performed using a Rever Tra Ace α-First Strand cDNA Synthesis Kit (TOYOBO, Osaka, Japan). Briefly, 1 μg of total RNA, oligo dT-primer, and dNTPs were incubated at 65℃ for 5 min, then 10 μL of cDNA synthesis mixture was added and the mixture was incubated at 50℃ for 50 min. The reaction was terminated by adding 1 μL of RNaseH and

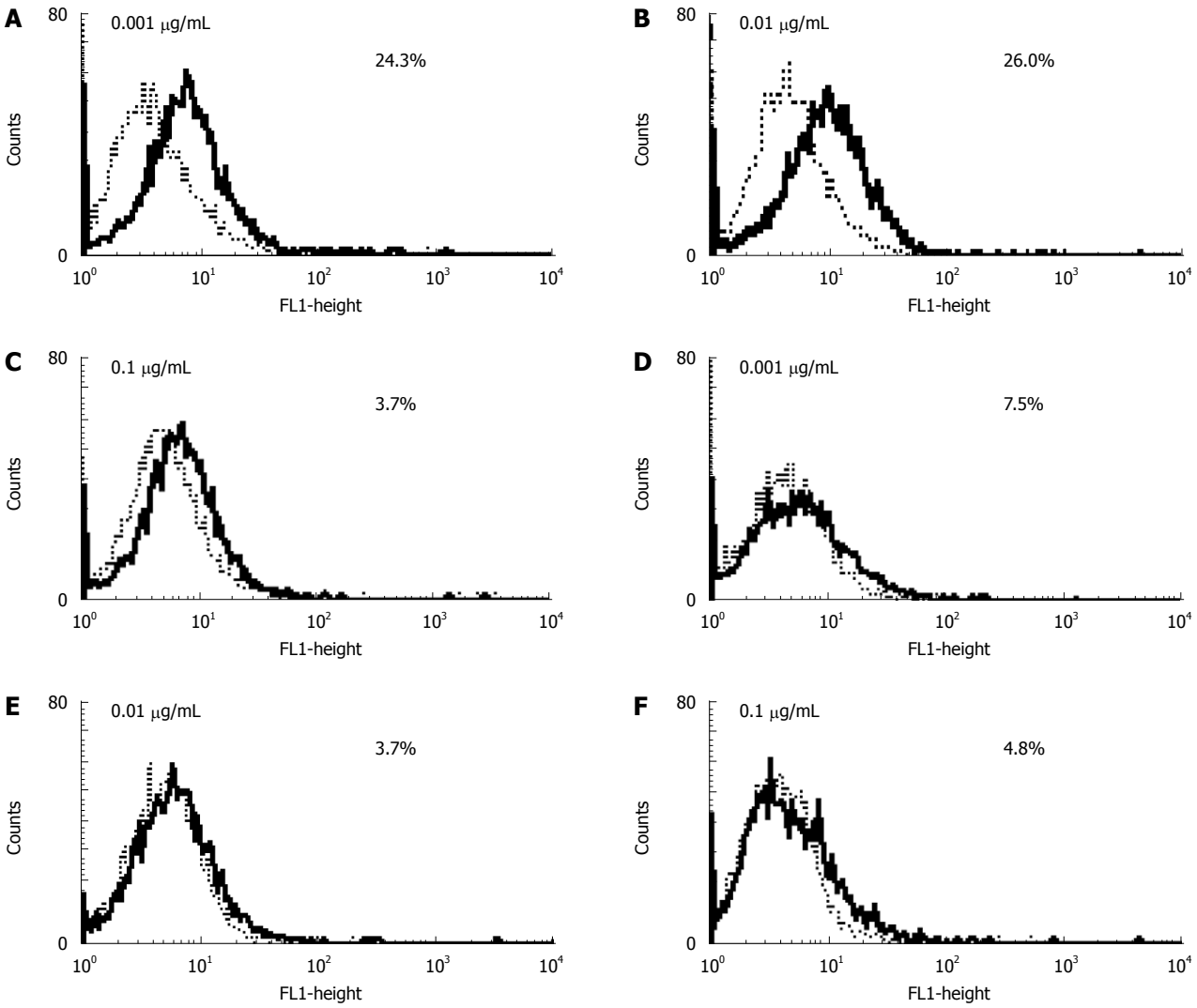

Figure 1 MK615 decreases receptor of advanced glycation end-product expression in HuH7 and HepG2 cells. Cells were plated at 1 × 10^5/well in 24-well plates and then cultured for 24 h. MK615 was then added to the medium, followed by culture for a further 24 h. MK615 (0.1 μg/mL) decreased RAGE expression in both HuH7 (A-C) and HepG2 (D-F).

incubating the mixture at 37℃ for 20 min.

The sequences of the primers were as follows: β-actin: sense-primer 5'-GGACTTCGAGCAAGAGATGG-3', anti-sense 5'-AGCACTGTGTTGGCGTACAG-3'; RAGE: sense-primer 5'-CACACTGCAGTCGGAGCTAA-3', anti-sense 5'-GCTACTGCTCCACCTTCTGG-3'. Conventional polymerase chain reaction (PCR) was performed with Hottaq mix (x,x). The PCR conditions consisted of 95℃ for 5 min, 40 cycles at 95℃ for 30 s, 62℃ for 30 s, and 60℃ for 30 s. The PCR products were separated on 1% agarose gel.

Real-time PCR was performed with an ABI Prism 7700 sequence detector (Applied Biosystems, Warrington, UK). The PCR reaction was carried out in a final volume of 25 μL, which included 2 μL cDNA, 12.5 μL 2 × SYBR Green (Applied Biosystems), 0.5 μL of 25 nmol/L sense and antisense primers, and H_2O. The PCR conditions consisted of 40 cycles at 95℃ for 30 s and 60℃ for 30 s. Samples were assayed in triplicate. Means and standard deviations were calculated from the data obtained. For each sample, at least three assays were performed. The *t* value was calculated as the mean of three different assays. The level of expression was calculated using the formula: Relative expression (*t*-value) = (Copy number of target molecule/Copy number of β-actin) × 1000[3,4].

Western blotting

Anti-RAGE antibodies (#ab54741) were purchased from Abcam Inc. (Cambridge, MA). After cells had been collected, they were washed twice with cold PBS, lysed with 200 μL of 0.5% (w/v) SDS, and centrifuged at 10000 r/min. The supernatants were adjusted by dilution so as to contain equal amounts of protein, as tested using a BCA Protein Assay Kit (Pierce, Rockford, IL). Samples (20 μg protein) were run on 12.5% (w/v) SDS-PAGE with 10% gel and electroblotted onto PVDF membranes. The blots were blocked for 1 h with 5% (w/v) non-fat milk powder and 0.1% (v/v) Tween 20 in Tris-NaCl, then exposed to the primary antibody at a 1000-fold dilution overnight at 4℃. After extensive washing, the blots were incubated

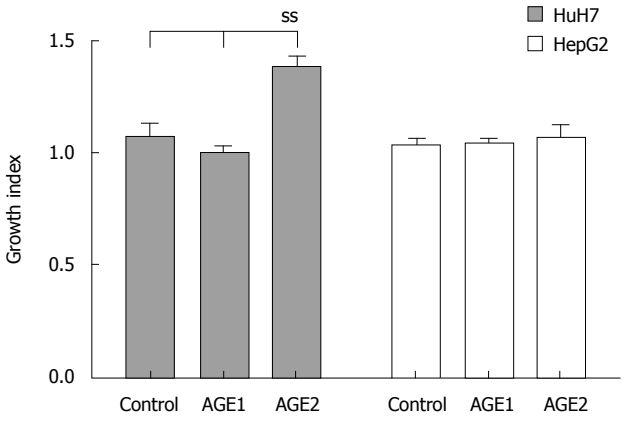

Figure 2 Proliferative effect of advanced glycation end-products on HuH7 and HepG2 cells. Cells were cultured for 24 h, and advanced glycation end-products (AGEs) (0.1 mg/mL) were then added. After 24 h, the MTT assay was performed. Glycer-AGE significantly stimulated proliferation of HuH7 cells. ss: Statistically significant.

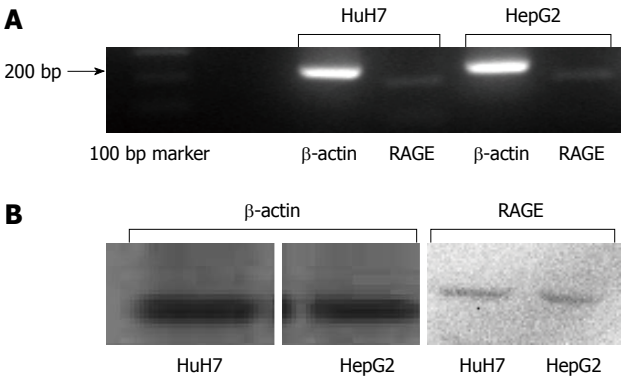

Figure 3 Expression of endogenous receptor of advanced glycation end-product in HuH7 and HepG2 cells. Reverse transcription-polymerase chain reaction (A) and Western blotting (B) showed that HuH7 and HepG2 expressed receptor of advanced glycation end-product (RAGE) endogenously. β-actin was used as a positive control. bp: Base pairs.

with the secondary horseradish-peroxidase-conjugated antibody (1:2000) for 2 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Amersham Life Sciences, Arlington Heights, IL)[2-4].

Flow cytometry

Anti-RAGE antibody was the same as that used in Western blotting. An isotype-matched antibody was purchased from Abcom. The cells were collected and stained with control or anti-RAGE monoclonal antibody for 1 h, washed twice with PBS, and then stained with FITC-conjugated anti-mouse IgG monoclonal antibody for 30 min. Then cells were again washed twice with PBS and analyzed using a FACSCalibur (Becton-Dickinson). The percentage shift was calculated by subtracting the histogram obtained with anti-RAGE monoclonal antibody from that obtained with the control antibody[23].

MTT assay

For the MTT assay, 15 μL MTT (5 g/L) was added to each well, and the cells were incubated for 4 h. Then, 100 μL solubilization solution/stop mix was added, in accordance with the manufacturer's recommendation, and the plates were left to stand for 60 min. The absorbance at 570 and 630 nm was then measured with an ELISA reader. The actual counts were calculated by subtracting the absorbance at 570 nm from that at 630 nm. Each assay was performed in triplicate and the average absorbance was calculated[2-4].

Growth index (Figure 2) was calculated as follows. Cells were plated out at 5 × 10^3 cells/well in 96-well plates and cultured for 24 h, then AGEs were added to the culture medium (Medium) at 0.1 mg/mL. After 24 h, the MTT assay was performed, and experiments were repeated 3 times. The mean values of the counts were calculated for each experiment. Growth index was calculated by dividing the mean for the control, Glc-AGE, or Glycer-AGE by the mean for the Medium. Standard deviation was calculated for all three experiments.

The percentage inhibition was calculated as follows. Two culture sets were set up, the cells being plated out at 5 × 10^3/well in 96-well plates, and cultured for 24 h. The cells were then cultured with Medium, control, Glc-AGE, or Glycer-AGE, and each well was cultured in quadruplicate. One plate was cultured without MK615 and the other with MK615 0.1 μg/mL. The percentage inhibition was calculated as: % inhibition = (count of cells with MK615 - count of cells without MK615) × 100/count without MK615. The experiments were repeated three times, and the means and standard deviations were calculated.

Apoptosis assay

Cells were cultured under control conditions, or with Glc-AGE, Glycer-AGE, or MK615 for 24 h, then harvested by trypsinization and washed twice with PBS. The annexin V binding assay was performed using an Annexin V-FITC Apoptosis Detection kit (Becton-Dickinson) in accordance with the supplier's instructions. At least 1 × 10^6 cells were incubated with FITC-conjugated annexin V at room temperature for 15 min, and the cells were then analyzed on a FACscan (Becton-Dickinson).

Statistical analysis

One-factor analysis of variance (ANOVA) with post-hoc test was used for comparison of the three subgroups by GraphPad Prism 5.0 (Graphpad Software, La Jolla, CA). The results obtained by flow cytometry were analyzed statistically with the Kolmogorov-Smirnov test using Cell Quest software (Becton-Dickinson)[23]. Differences at $P < 0.05$ were accepted as significant. Statistical analysis of percentage growth inhibition was performed using the unpaired, two-tailed *t* test.

RESULTS

Results of RT-PCR (Figure 3A) and Western blotting (Figure 3B) showed that HuH7 and HepG2 expressed RAGE endogenously. Flow cytometry with anti-RAGE

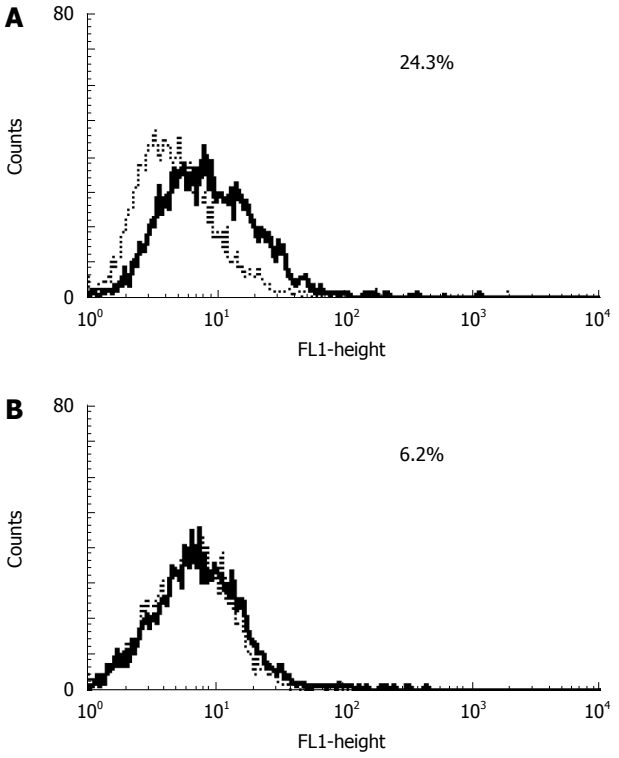

Figure 4 Cell surface expression of receptor of advanced glycation end-product in HuH7 and HepG2 cells. Flow cytometry showed receptor of advanced glycation end-product expression in both HuH7 (A) and HepG2 (B).

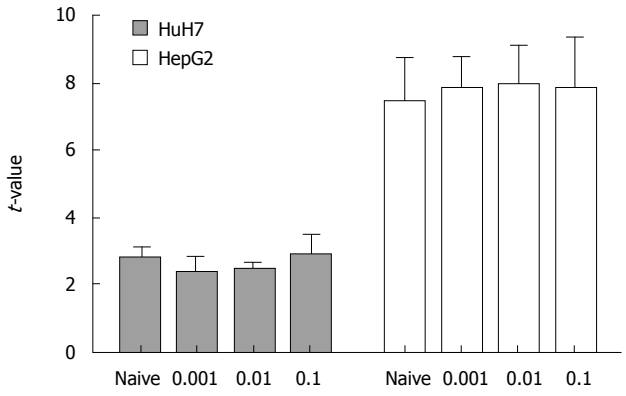

Figure 5 MK615 does not decrease receptor of advanced glycation end-product mRNA. The concentration of receptor of advanced glycation end-product mRNA did not differ between MK615-treated HuH7 and HepG2 cells.

antibody staining demonstrated the expression of membrane-bound RAGE in both HuH7 and HepG2 cells at 24.3% and 6.2%, respectively (Figure 4A and B).

Next, we investigated the effect of MK615 on the cell surface expression of RAGE in HuH7 and HepG2 cells. Cells were incubated with different concentrations of MK615. In HuH7, the expression levels of RAGE at MK615 concentrations of 0.001, 0.01, and 0.1 μg/mL were 24.3%, 26.0%, and 3.7%, respectively (Figure 1A-C), whereas the corresponding levels in HepG2 were 7.5%, 3.7%, and 4.8%, respectively (Figure 1D-F).

The results of real-time PCR using samples of RNA obtained under the same conditions as those for flow cytometry showed that transcription of RAGE mRNA did not differ between MK615-treated and non-treated cells (Figure 5).

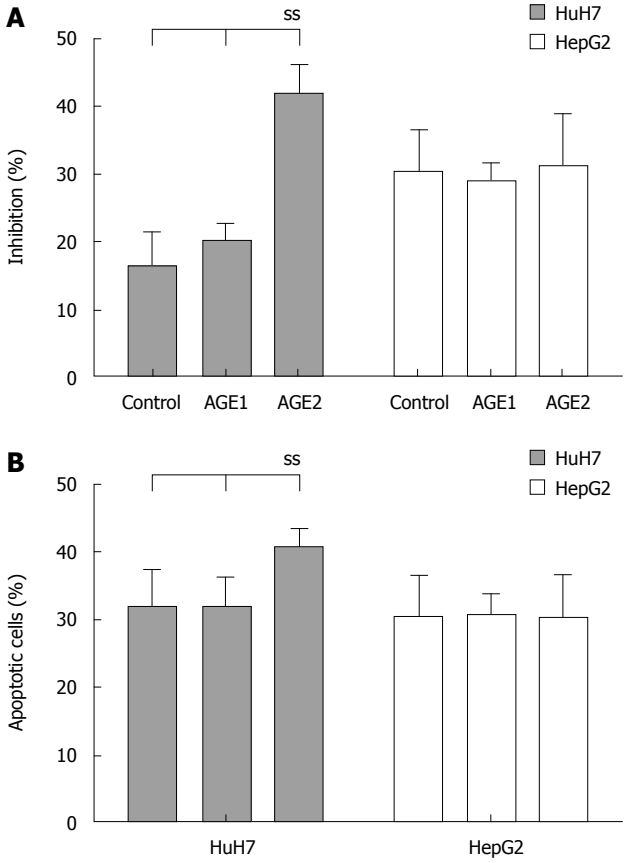

Figure 6 Suppression of advanced glycation end-products-stimulated growth by MK615 and apoptosis induction. A: The percentage inhibition of Glycer-advanced glycation end-products (AGE) by MK615 was significantly higher than those for the control, and Glc-AGE in HuH7 cells. In HepG2 cells, there were no significant differences among the groups. B: Frequency of apoptotic cells of Glycer-AGE by MK615 was significantly higher than those for the control, and Glc-AGE in HuH7 cells. In HepG2 cells, there were no significant differences among the groups. ss: Statistically significant.

To evaluate the stimulation of growth by AGEs, HuH7 and HepG2 cells were incubated with AGEs (Figure 5). The growth indices of the unglycated BSA control, Glc-AGE, and Glycer-AGE were 1.06 ± 0.08, 0.99 ± 0.04, and 1.38 ± 0.05 in HuH7, and 1.03 ± 0.04, 1.04 ± 0.03, and 1.07 ± 0.05 in HepG2, respectively. In HuH7, the growth index of Glycer-AGE was significantly higher than those of the control and Glc-AGE ($P = 0.037$). In HepG2, there were no significant differences in growth index among the three treatments ($P = 0.905$).

Figure 6A shows the suppression of AGEs growth stimulation by MK615. The percentages of inhibition for the Medium, control, Glc-AGE, and Glycer-AGE in HuH7 cells were 29.0% ± 3.5%, 16.2% ± 5.1%, 20.1% ± 2.7%, and 41.7% ± 4.1%, respectively. The percentage inhibition of Glycer-AGE-induced growth stimulation was significantly higher than that for Medium, Control, or AGE1 ($P = 0.02$ by one-way ANOVA). In HepG2 cells, the percentages of inhibition for the Medium, control, Glc-AGE, and Glycer-AGE were 28.9% ± 8.1%, 30.1%

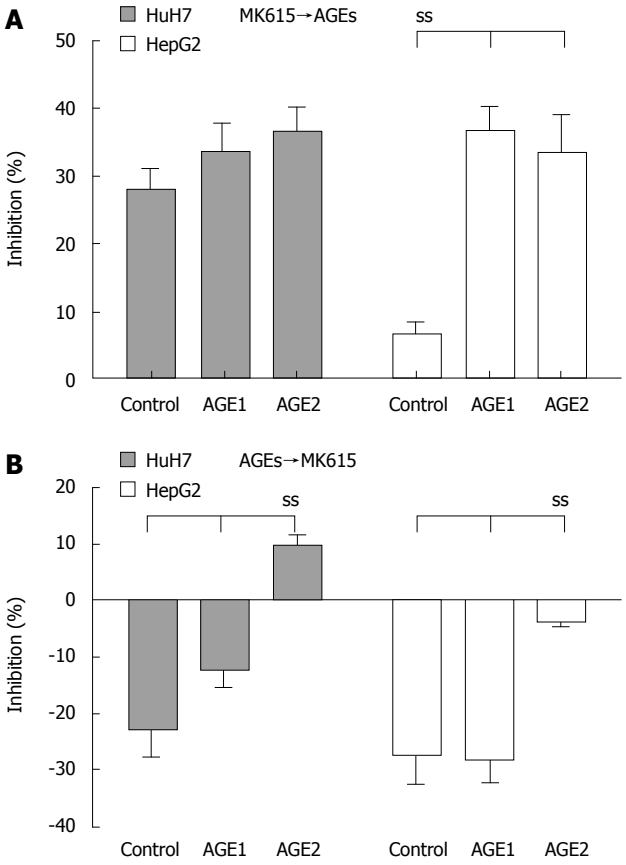

Figure 7 Changes in percentage inhibition resulting from pre- or post-treatment with MK615. A: Cells were cultured for 24 h with MK615, and then advanced glycation end-products (AGEs) were added, followed by culture for a further 24 h. The proliferative effect of Glycer-AGE was abrogated by pretreatment with MK615 in both HuH7 and HepG2 cells; B: Cells were cultured for 24 h with AGEs, and then MK615 were added, followed by culture for a further 24 h. The proliferative effect elicited by Glycer-AGE was still evident, but was reduced. ss: Statistically significant.

± 6.3%, 29.0% ± 2.7% and 31.1% ± 7.9%, respectively. There were no significant differences among the groups (P = 0.18 by one-way ANOVA). For HuH7, the frequencies of apoptotic cells for the control, Glc-AGE, and Glycer-AGE were 31.5% ± 5.7%, 31.3% ± 4.9%, and 40.3% ± 3.1%, respectively (P = 0.04 by one-way ANOVA), and those for HepG2 were 30.2% ± 6.2%, 30.5% ± 3.1%, and 30.1% ± 6.3%, respectively (P = 0.12 by one-way ANOVA) (Figure 6B).

Figure 7A shows the percentages of inhibition when the cells were cultured with AGEs following 24 h of culture with MK615. In HuH7 cells, the percentages of inhibition for the control, Glc-AGE, and Glycer-AGE were 27.9% ± 3.2%, 33.4% ± 4.5%, and 36.3% ± 4.1%, respectively (P = 0.08 by one-way ANOVA), and the corresponding values in HepG2 were 6.7% ± 1.9%, 36.6% ± 3.7% and 30.1% ± 6.3%, respectively (P = 0.03 by one-way ANOVA). Figure 7B shows the percentages of inhibition when the cells were cultured with MK615 following 24 h of culture with AGEs. In HuH7 cells, the percentages of inhibition for the control, Glc-AGE, and Glycer-AGE were -22.3% ± 5.1%, -12.3% ± 3.2%, and 9.6% ± 2.2%, respectively (P = 0.02 by one-way ANOVA), and the corresponding values for HepG2 were -27.5% ± 5.9%, -27.8% ± 4.7%, and -3.9% ± 0.8%, respectively (P = 0.02 by one-way ANOVA).

DISCUSSION

HuH7 and HepG2 cells showed surface expression of RAGE, but the level of expression differed between them. A previous study reported that HepG2 did not express RAGE[24], although the present study confirmed weak expression of RAGE in HepG2 at both the mRNA and protein level. Flow cytometry revealed that HuH7 cells expressed RAGE at a higher level than did HepG2. On the other hand, results of real-time PCR (Figure 5) showed higher t-values for HepG2 than for HuH7. Because we calculated the expression of RAGE mRNA relative to β-actin mRNA, the relative expression of RAGE mRNA would have been influenced by the expression level of β-actin mRNA. However, the reason for the discrepancy in the expression of RAGE mRNA between flow cytometry and real-time PCR was unclear. Although both cell lines were derived from well differentiated HCC, HuH7 was established from a Japanese patient, whereas HepG2 was from a Caucasian. Hiwatashi *et al*[24] reported that expression of RAGE was lower in normal liver and hepatitis, and higher in HCC. Although the mechanism responsible for the difference in RAGE expression is not clear, and the level at which RAGE expression stimulates the growth of HCC was not determined, the difference would have been related to the response of the cells to AGEs, as described below.

HuH7 showed a statistically significant growth response to Glycer-AGE, but not to control or Glc-AGE. HepG2, which expressed a low level of RAGE, did not show any significant growth response to control, Glc-AGE, or Glycer-AGE (Figure 2). Our results indicated that Glycer-AGE stimulated the growth of HuH7 cells, which show higher surface expression of RAGE, whereas the degree of stimulation was not marked in HepG2 cells, which show low surface expression of RAGE. Furthermore, the control and Glc-AGE exerted no proliferative effect on either of the cell lines. It has been difficult to study the biological effects of AGEs because of their heterogeneity. As CML, pyrroline, and Glc-AGE are not as toxic as Glycer-AGE, it is necessary to obtain data using a representative TAGE, of which Glycer-AGE is one of the more toxic forms. Glycer-AGE, but not Glc-AGE, CML, and pyrroline, has strong binding affinity for RAGE and subsequently elicits oxidative stress and vascular inflammation, being implicated in the accelerated atherosclerosis seen in patients with diabetes mellitus[25,26]. Recently, we have also demonstrated that Glycer-AGE plays an important role in the pathogenesis of angiopathy in diabetic patients[27,28]. Moreover, there is a growing body of evidence to suggest that the interaction of TAGE with RAGE elicits oxidative stress in numerous types of cells, thereby possibly contributing to the pathological changes that characterize the vascular complications of diabetes. Glycer-AGE-RAGE interaction triggers the generation of NADPH oxidase-mediated reactive oxygen species (ROS),

and subsequently activates p38/MAP kinase and NF-κB, resulting in cell proliferation[27,28].

As shown in Figure 1, MK615 decreased the expression of RAGE on HuH7 cells at a concentration of 0.1 μg/mL, whereas the effect on HepG2 cells was less marked. This decrease in the expression of RAGE was not due to suppression of transcription, because the results of real-time PCR revealed that the transcripts of RAGE mRNA did not differ between naïve and MK615-treated HuH7 and HepG2 (Figure 5). It remains to be clarified whether the decrease in the cell-surface expression of RAGE is due to solubilization of RAGE, degradation, or other mechanisms.

When cells were cultured with AGEs and MK615 simultaneously, MK615 was found to inhibit the growth effect of Glycer-AGE (Figure 6), and when the cells were cultured with AGEs following exposure to MK615, the growth effect elicited by Glycer-AGE was abrogated (Figure 7A). Furthermore, when the cells were cultured with AGEs followed by MK615, the growth effect elicited by Glycer-AGE was still evident, but was reduced (Figure 7B). MK615 inhibits the activation of MAPK/NF-κB[15,16], which is thought to be a major pathway of the AGEs/RAGE system. Because MK615 contains several triterpenoids and other unknown substances, the mechanism responsible for abrogation of the proliferative effect of Glycer-AGE on HCC cells might be multifactorial. However, the decrease of RAGE expression elicited by MK615 may play a crucial role in suppressing the Glycer-AGE-induced proliferation of HCC cells.

In conclusion, Glycer-AGE (a representative TAGE) exerts a potent proliferative effect on HuH7 cells, which express a high level of RAGE on their surface. The effect of RAGE may be partly dependent on the degree of cell-surface RAGE expression. MK615 inhibits Glycer-AGE-induced proliferation of HuH7 cells by decreasing their cell surface expression of RAGE. Our present results suggest that further studies are warranted to explore the role of Glycer-AGE in HCC, and to examine the therapeutic effect of MK615.

COMMENTS

Background

This study focused on two topics: advanced glycation-end products (AGEs), and MK615, an extract from the Japanese apricot, *Prunus mume* Sieb. et Zucc (*ume* in Japanese). AGEs are the products of non-enzymatic, irreversible glycation of proteins, and the causative agents of various disorders, including inflammation and cancers. AGEs constitute a heterozygous group, and recent studies have revealed that most of them are not really toxic, although glyceraldehyde-derived AGE (Glycer-AGE) is a toxic AGE (TAGE). In the present study, we used purified Glycer-AGE. AGEs bind to the cell-surface receptor of AGE (RAGE) to exert biological effects. It has been shown that MK615 exerts anti-inflammatory affects by modifying the expression of RAGE. Thus, AGEs and MK615 are connected *via* RAGE.

Research frontiers

Glycer-AGE is a real TAGE, and a limited number of studies have been conducted to clarify the role of Glycer-AGE in cancer. As mentioned before, AGEs are a heterogenous group of products, and future studies should focus on AGEs using Glycer-AGE. As shown in the present study, Glycer-AGE exerts stronger effects than glucose-derived AGE. The biological effects of Glycer-AGE remain to be elucidated.

Innovations and breakthroughs

Glycer-AGE potently stimulated the growth of hepatocellular carcinoma (HCC) cells, and the effect was inhibited by MK615. This suggests that Glycer-AGE (TAGE) stimulates the proliferation of HCC, and that MK615 may have a therapeutic effect on this activity.

Applications

Clinically, MK615 ameliorates hepatitis due to hepatitis C virus. Although the mechanism remains unclear, MK615 may exert its effect by downregulating the expression of RAGE. Although further studies will be needed, MK615 may exert a therapeutic effect on HCC by inhibiting Glycer-AGE-dependent cancer growth.

Terminology

MK615 is an extract from the Japanese apricot, *Prunus mume* Sieb. et Zucc (*ume* in Japanese). AGEs are products of the non-enzymatic, irreversible glycation of proteins. RAGE is a cell-surface receptor of AGEs. TAGE is a toxic AGE that is derived from glyceraldehyde (Glycer-AGE).

Peer review

The study is focused on the interesting topic of RAGE-dependent cellular proliferation. The authors demonstrate semi-functional data that RAGE could be of relevance in hepatocellular tumour growth.

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BRIEF ARTICLE

Comparative study of therapeutic effects of PPI and H2RA on ulcers during continuous aspirin therapy

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Abstract

AIM: To compare the therapeutic effects of proton pump inhibitors (PPI) and histamine 2 receptor antagonists (H2RA) on gastroduodenal ulcers under continuous use of low-dose aspirin.

METHODS: Sixty patients who had a gastroduodenal ulcer on screening endoscopy but required continuous use of low-dose aspirin were randomly assigned to receive PPI (lansoprazole 30 mg, *n* = 30) or H2RA (famotidine 40 mg or if famotidine had been administered before assignment, ranitidine 300 mg, *n* = 30). The therapeutic effects were evaluated by endoscopy after 8-wk treatment. The presence or absence of *Helicobacter pylori* (*H. pylori*) was determined by urea breath test before treatment. Abdominal symptoms were compared with the gastrointestinal symptom rating scale (GSRS) questionnaire before and after treatment.

RESULTS: Twenty-six patients in the PPI group and 26 patients in the H2RA group, excluding dropouts, were analyzed. There were no significant differences in median age, sex, underlying disease, smoking status, *H. pylori* infection, prevalence of ulcers before treatment, and lesion site between the two groups. The therapeutic effects were endoscopically evaluated as healed in 23 patients (88.5%) and not healed in 3 patients in the PPI group and as healed in 22 patients (84.6%) and not healed in 4 patients in the H2RA group. Abdominal symptoms before treatment were uncommon in both groups; the GSRS scores were not significantly reduced after treatment as compared with before treatment.

CONCLUSION: The healing rate of gastroduodenal ulcers during continuous use of low-dose aspirin was greater than 80% in both the PPI group and the H2RA group, with no significant difference between the two groups.

Key words: Low-dose aspirin; Proton pump inhibitors; Histamine 2 receptor antagonists; Gastric ulcer

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Nema H, Kato M. Comparative study of therapeutic effects of PPI and H2RA on ulcers during continuous aspirin therapy. *World J Gastroenterol* 2010; 16(42): 5342-5346 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i42/5342.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i42.5342

INTRODUCTION

As the population of Japan ages, the use of low-dose aspirin has increased to prevent cerebral and myocardial infarction. Many reports from Japan and overseas showing evidence that low-dose aspirin is useful for prevent-

ing thrombosis have been published. However, low-dose aspirin use raises concern about its adverse effects such as gastrointestinal mucosal injury[1,2]. Low-dose oral aspirin (300 mg or less) is reportedly associated with a 2.6- to 3.0-fold increased risk of ulcers[3] and a 1.59-fold increased risk of gastrointestinal bleeding[4]. The prevalence of low-dose (200 mg or less) aspirin-associated gastroduodenal ulcers was 11.9% to 15.7% in Japanese patients treated for ischemic heart disease[5], and another case-control study has shown that low-dose aspirin is associated with a 5.5-fold increased risk of gastrointestinal bleeding[6]. However, low-dose aspirin is administered for the purpose of secondary prevention of cardiovascular events, and because drug suspension due to gastrointestinal injury would increase the risk of thrombosis, it is frequently difficult to discontinue the use of low-dose aspirin. In fact, when long-term users of low-dose aspirin suspended the use of the drug, the risk of thrombosis increased[7,8]. Therefore, gastrointestinal injury should be treated under continuous use of low-dose aspirin. Previous studies from Western countries have shown that proton pump inhibitors (PPI) are first-line drugs for the treatment of gastrointestinal injury associated with non-steroidal anti-inflammatory drugs (NSAIDs) including aspirin[9-11]. However, there are no prospective studies focusing on low-dose aspirin, nor are there studies from Japan where *Helicobacter pylori* (*H. pylori*) infection rate is high.

We conducted a prospective, multicenter controlled study to compare the therapeutic effects of ordinary dose of PPI and histamine 2 receptor antagonists (H2RA) on gastroduodenal ulcers during continuous use of low-dose aspirin.

MATERIALS AND METHODS

Patients

Two hundred twenty-nine low-dose aspirin users developed an endoscopy-proven gastrointestinal ulcer between May 2006 and November 2009 at the Hokkaido University Hospital and associated facilities. Out of these patients, 78 patients who did not meet inclusion criteria were excluded. Patients were excluded from the study if they had gastrointestinal bleeding as a complication, underwent gastrectomy, had a serious complication, had been taking non-aspirin NSAIDs regularly, or were under 20 years or over 80 years old. Patients who administered non-aspirin NSAIDs on an as-needed basis were included.

One hundred fifty-one patients were recruited to this study. All of them wished to continue taking low-dose aspirin and gastric acid secretion inhibitors, because they had no vascular events after aspirin therapy. However, the majority of patients rejected a second endoscopy after 8 wk. Finally, 60 patients who provided written informed consent and required continuous use of low-dose aspirin were entered into the study.

This study was approved by the ethics committee of each facility. Written informed consent was obtained from each participant in this study.

Methods

Enrolled patients were randomly assigned to the PPI group (lansoprazole 30 mg, $n = 30$) or the H2RA group (famotidine 40 mg, $n = 30$) by Central Registry *via* the Internet. If patients who had been treated with famotidine before randomization were assigned to the H2RA group, they were treated with ranitidine 300 mg instead.

The presence of *H. pylori* was determined by urea breath test before treatment. An exhaled-breath sample was collected 20 min after patients took ^{13}C-urea 100 mg orally, and the cut-off value was set at Δ^{13}C 2.5‰[12].

Therapeutic effects were based on endoscopic findings obtained at the end of 8 wk treatment. Endoscopy was performed before and after treatment by a single endoscopist at each facility using GIF-XQ 240 (Olympus Corporation, Tokyo, Japan). Mucosal defects were measured with biopsy forceps and an ulcer was defined as a mucosal defect when it was 3 mm or more in diameter. Photographs of lesions were taken before and after treatment and therapeutic effects were evaluated by a single physician. Complete disappearance of a mucosal defect was defined as healed, reduction of mucosal defect as reduced, no change in mucosal defect as unchanged, enlargement of mucosal defect as aggravated.

Patients were instructed to record abdominal symptoms using gastrointestinal symptom rating scale (GSRS) just before the first and the second endoscopic examinations. The GSRS scores were compared before and after treatment to evaluate the improvement of abdominal symptoms.

Statistical analysis

Endoscopic healing rate and self-improvement rate using GSRS were statistically determined by Wilcoxon test. The statistical software used was the SPPS 15.0. A level of $P < 0.05$ was considered statistically significant.

RESULTS

Four patients in the PPI group and 4 patients in the H2RA group dropped out of the study because they refused to undergo endoscopy when their symptoms disappeared or they were moved to another hospital. Medication compliance rate was as high as 80% or more among patients excluding dropouts. Twenty-six patients in the PPI group and 26 in the H2RA group qualified for analysis.

Buffered aspirin tablets (Bufferin 81) and enteric coated tablets (Bayaspirin 100) were continuously used by 11 and 15 patients, respectively, in the PPI group and by 10 and 16 patients, respectively, in the H2RA group. Two patients in each group had used NSAIDs as needed for headache (diclofenac sodium in 3 patients and zaltoprofen in 1 patient). H2RA had been used before enrollment in 3 patients in the PPI group (usual dose of ranitidine, usual dose of nizatidine, and half dose of famotidine respectively) and in 4 patients in the H2RA group (usual dose of famotidine, usual dose of ranitidine, usual dose of nizatidine, and half dose of nizatidine respectively). No patients

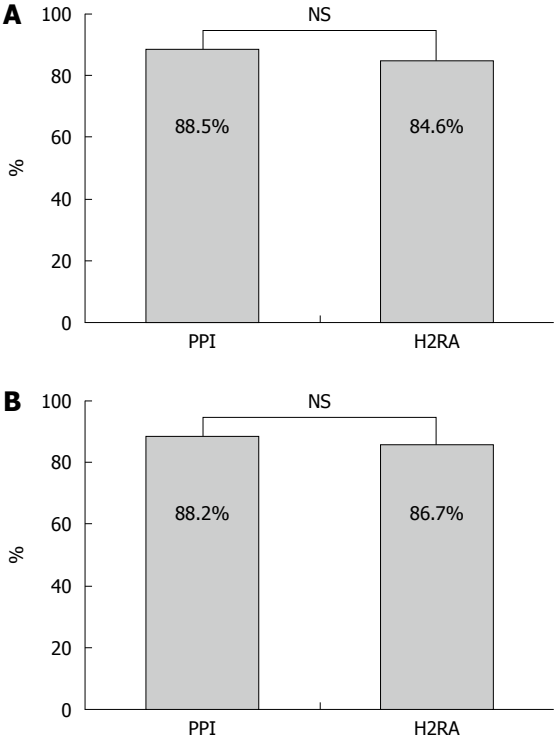

Figure 1 Gastroduodenal ulcer healing was endoscopically achieved in proton pump inhibitors and histamine 2 receptor antagonists group patients. A: Gastroduodenal ulcer healing was endoscopically achieved in 88.5% of proton pump inhibitors (PPI) group patients and in 84.6% of histamine 2 receptor antagonists (H2RA) group patients. There was no significant difference between the two groups; B: Gastroduodenal ulcer healing in patients with non-pangastritis was endoscopically achieved in 88.2% of PPI group and in 86.7% of H2RA group. There was no significant difference between the two groups. NS: Not significant.

Table 1 Background of patients *n* (%)

	PPI group (*n* = 26)	H2RA group (*n* = 26)	*P* value
Median age (yr)	67.2 ± 8.7	71.1 ± 6.9	NS
Male	19 (73.1)	19 (73.1)	NS
Ischemic heart disease	15 (57.7)	13 (50.0)	NS
Smoking	11 (42.3)	8 (30.8)	NS
Helicobacter pylori (+)	13 (50.0)	12 (46.2)	NS
Ulcer size > 5 mm	13 (50.0)	12 (46.2)	NS
Location of mucosal defect			
Stomach	24	23	NS
Duodenal	2	3	
Aspirin			
Buffered	10	10	NS
Enteric-coated	16	16	

PPI: Proton pump inhibitors; H2RA: Histamine 2 receptor antagonists; NS: Not significant.

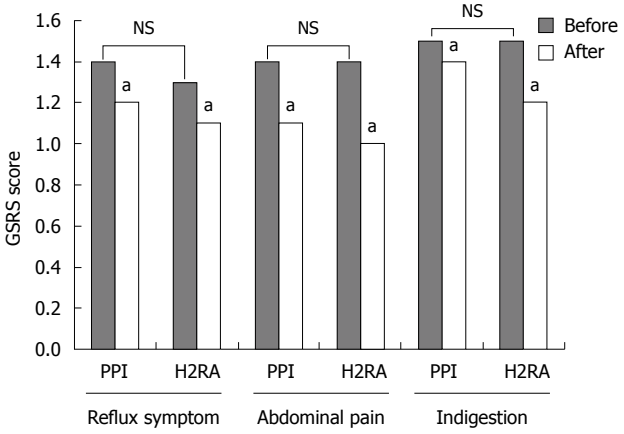

Figure 2 Improvement of abdominal symptoms was evaluated using gastrointestinal symptom rating scale scores. The scores were not significantly reduced after treatment as compared with before treatment in both proton pump inhibitors (PPI) and histamine 2 receptor antagonists (H2RA) groups. [a]NS *vs* before. NS: Not significant.

had used PPI before enrollment. If patients assigned to the H2RA group had a history of famotidine use, they were administered ranitidine 300 mg.

There were no significant differences in median age, sex, underlying disease, smoking status, *H. pylori* infection, prevalence of ulcers before treatment, or lesion site between the PPI group and the H2RA group (Table 1).

The therapeutic effects were endoscopically evaluated as healed in 23 of 26 patients in the PPI group and in 22 of 26 patients in the H2RA group, with no significant difference between the groups (Figure 1A).

Three patients in the PPI group were evaluated as not healed, including 2 evaluated as reduced and 1 evaluated as unchanged. In the 2 patients evaluated as reduced, multiple ulcers were observed at the antrum of the stomach and ulcers 10 and 5 mm in maximum diameter were reduced to 3 and 2 mm, respectively, after treatment. In the patient evaluated as unchanged, the use of 100 mg aspirin enteric coated tablets was continued for the treatment of angina pectoris, endoscopy revealed a solitary ulcer 5 mm in diameter at the body of the stomach, and there was no evidence of *H. pylori* infection.

Four patients in the H2RA group were evaluated as not healed, including 3 evaluated as reduced and 1 evaluated as unchanged. In the 3 patients evaluated as reduced, solitary ulcers 15 mm at the antrum of the stomach, 15 mm at the body of the stomach, and 3 mm at the antrum of the stomach were all reduced to 2 mm after treatment. In the patient evaluated as unchanged, the use of 162 mg buffered aspirin tablets was continued for the treatment of old myocardial infarction, multiple ulcers were observed at the antrum of the stomach 3 mm in maximum diameter on endoscopy, there was no evidence of *H. pylori* infection, diclofenac sodium was prescribed as needed, and usual dose of ranitidine had been administered before assignment to treatment group.

Improvement of abdominal symptoms was evaluated after treatment by comparing the GSRS scores before and after treatment. Pretreatment scores indicated that abdominal symptoms were uncommon in both the PPI group and the H2RA group. The scores were not significantly reduced after treatment as compared with before treatment in either group (Figure 2). No side events were observed in either group.

For research about influence of acid secretion, both groups were subdivided into 2 groups based on whether the patient had pangastritis or not. There were 17 patients in the PPI group with pangastritis and 15 in the H2RA

group. The therapeutic effects were not significantly different between the non-pangastritis groups (Figure 1B).

DISCUSSION

One of the major adverse events associated with low-dose aspirin is gastrointestinal bleeding. However, screening endoscopy is widely used in Japan and peptic lesions are frequently pointed out on screening endoscopy before the development of overt gastrointestinal bleeding. Therefore, the subjects of this study were those found to have gastroduodenal lesions on endoscopy without gastrointestinal bleeding as a complication.

Low-dose aspirin-induced mucosal defect is commonly 5 mm or less[3]. Considering that lesions 5 mm or less in size can cause bleeding, and if left untreated, may be enlarged, we believe that small ulcers should be treated. Thus, patients with ulcers 3 mm or more were included in this study.

Controlled studies of the therapeutic effects of PPI *vs* H2RA for NSAIDs-associated gastric ulcers have shown that the healing rate after 8 wk treatment was significantly higher in the PPI group than in the H2RA group[9-11].

A recent controlled study investigated the preventative effects of PPI *vs* H2RA on the occurrence of bleeding ulcers associated with low-dose aspirin in patients not infected with *H. pylori* and concluded that PPI are significantly more effective than H2RA in preventing the occurrence of bleeding ulcers and abdominal symptoms[13]. However, the results of their preventative effects against ulcers cannot be extrapolated to the healing of ulcers. On the other hand, it was reported that patients who take H2RA with low-dose aspirin had fewer peptic ulcers than patients who take placebo[14].

In the present study, the healing rate of gastroduodenal ulcers associated with low-dose aspirin was similar in the PPI group and the H2RA group. This may be explained by the facts that half of the subjects included in this study were infected with *H. pylori,* the subjects were limited to the Japanese, and they had a lower ability to secrete gastric acid. Previous studies involving patients with duodenal ulcers have shown that average maximum gastric acid secretion was 21.9 mEq/h for Japanese men and 43.2 mEq/mL for American men[15,16]. However, a retrospective study from Western countries investigated the effects of H2RA (famotidine 20-40 mg or ranitidine 150-300 mg) and PPI (omeprazole 20 mg) on lowering the risk of gastrointestinal bleeding in low-dose aspirin users and concluded that both drug classes have similar effects on preventing bleeding[17]. Further studies are needed to evaluate the therapeutic effects of both PPI and H2RA on low-dose aspirin-induced ulcers.

One patient evaluated as unchanged in the H2RA group developed an ulcer 3 mm in diameter, was not infected with *H. pylori*, and had a history of diclofenac sodium on an as-needed basis. One other patient in the PPI group had used diclofenac sodium as needed before assignment, but the ulcer was healed after 8 wk treatment. PPI are also reported to be effective when combined with low-dose aspirin and NSAIDs[18]. The combined use of low-dose aspirin and NSAIDs is known to be associated with an increased risk of gastrointestinal bleeding[3]. When low-dose aspirin is used alone, H2RA is expected to heal ulcers, but it may be better to choose PPI in high risk cases in which low-dose aspirin and NSAIDs are combined. Even in these cases, PPI are considered to be more effective than H2RA for 4 wk treatment[11,18].

The patient evaluated as unchanged in the PPI group had an ulcer 5 mm in size and was not infected with *H. pylori*. This case indicated that some ulcers are not healed even when treated with PPI. Such cases require further investigation for their appropriate treatment.

In this study, none of the patients were treated with combined aspirin and other antiplatelet drugs. However, recently use of combinations of low-dose aspirin and other antiplatelet drugs (e.g. clopidogrel and ticlopidine) have been increased, especially after coronary bypass graft surgery. PPI decrease clopidogrel's inhibitory effect on platelets[19]. It may be better to choose H2RA when low-dose aspirin and clopidogrel are combined. On the other hand, clopidogrel is not associated with an increased risk of gastrointestinal bleeding when used alone, but is associated with a 7.7-fold increased risk when used in combination with low-dose aspirin[20]. Further studies are required to investigate the preventive and therapeutic effects of PPI and H2RA on gastrointestinal events in the presence of combined aspirin and other antiplatelet drugs.

In conclusion, the healing rate of gastroduodenal ulcers was greater than 80% after 8-wk treatment with PPI or H2RA during continuous use of low-dose aspirin, with no significant difference between the two groups.

COMMENTS

Background

The use of aspirin has increased in the aging population. Aspirin increases the risk of gastrointestinal injury. The strategy to treat ulcers during low-dose aspirin treatment is not clear.

Research frontiers

It was reported that proton pump inhibitors (PPI) are more effective than histamine 2 receptor antagonists (H2RA) in prevention of aspirin-induced ulcers.

Innovations and breakthroughs

In this study, the healing rate of aspirin-induced ulcers was greater than 80% in both the PPI and the H2RA groups, with no significant difference between groups.

Applications

This study may be useful for considering changes in treatment of aspirin-induced ulcer.

Peer review

This article demonstrates some interesting points that compare the effects of PPI and H2-blocker in patients taking continuous low dose aspirin. The paper is well written, design is appropriate for end-points stated and patient number is nearly enough to draw conclusions.

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BRIEF ARTICLE

Endoscopic management of occluded metal biliary stents: Metal *versus* 10F plastic stents

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Abstract

AIM: To compare the efficacy of self-expandable metal stents (SEMSs) with 10F plastic stents (PSs) in the endoscopic management of occluded SEMSs.

METHODS: We retrospectively reviewed the medical records of 56 patients who underwent SEMS insertion for palliation of unresectable malignant biliary obstruction between 2000 and 2007 and subsequent endoscopic retrograde biliary drainage (ERBD) with SEMS or PS for initial SEMS occlusion between 2000 and 2008.

RESULTS: Subsequent ERBD with SEMS was performed in 29 patients and with PS in 27. The median time to stent occlusion after subsequent ERBD was 186 d in the SEMS group and 101 d in the PS group (P = 0.118). Overall median stent patency was 79 d for the SEMS group and 66 d for the PS group (P = 0.379). The mean number of additional biliary drainage procedures after subsequent ERBD in patients that died (n = 50) during the study period was 2.54 ± 4.12 for the SEMS group and 1.85 ± 1.95 for the PS group (P = 0.457). The mean total cost of additional biliary drainage procedures after the occlusion of subsequent SEMS or PS was $410.04 ± 692.60 for the SEMS group and $630.16 ± 671.63 for the PS group (P = 0.260). Tumor ingrowth as the cause of initial SEMS occlusion was the only factor associated with a shorter time to subsequent stent occlusion (101 d for patients with tumor ingrowth *vs* 268 d for patients without tumor ingrowth, P = 0.008).

CONCLUSION: Subsequent ERBD with PSs offered similar patency and number of additional biliary drainage procedures compared to SEMSs in the management of occluded SEMS.

Key words: Stents; Biliary tract neoplasms; Obstructive jaundice; Endoscopy; Endoscopic retrograde cholangiopancreatography

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INTRODUCTION

Endoscopic retrograde biliary drainage (ERBD) is now widely accepted as the standard intervention for the relief of obstructive jaundice in patients with unresectable malignant biliary obstruction[1-3]. Although plastic stents (PSs) were developed earlier, self-expandable metal stents (SEMSs) are now used widely as the initial choice for ERBD in this setting, as SEMSs offer longer patency[4-8]. Although it was suggested that a PS-based biliary drainage strategy may be more economical if the cost of endoscopic retrograde cholangiopancreatography (ERCP) is low relative to that of a SEMS[9,10], our recent retrospective study concluded that a SEMS-based biliary drainage strategy might offer better palliation without a significant increase in drainage-related medical cost, even where the cost of ERCP is low[11].

However, SEMSs do become occluded in some patients. Even covered SEMSs, which were developed to overcome stent occlusion caused by tumor ingrowth, become occluded due to tumor overgrowth, sludge, or migration[12,13]. There are a limited number of reports regarding the management of occluded SEMS, with various results[14-17]. The aim of this study was to compare the efficacy of SEMSs with 10F PSs in subsequent ERBD after the occlusion of initial SEMSs.

MATERIALS AND METHODS

Patients

Patients who underwent SEMS insertion (either endoscopically or percutaneously) for the palliation of unresectable malignant biliary obstruction at Seoul National University Hospital and the National Cancer Center between January 2000 and December 2007, and subsequent ERBD with SEMS or 10F PS for initial SEMS occlusion between January 2000 and December 2008, were evaluated. Patients were excluded when the initial SEMS was occluded within seven days of placement, when the follow-up period after subsequent ERBD was less than eight weeks without documented stent occlusion or patient death, or when endoscopic nasobiliary drainage or a percutaneous transhepatic biliary drainage (PTBD) was performed before the subsequent ERBD. The medical records were reviewed; endoscopic and radiological findings were studied to compare the stent patency and survival of the patients. Additional information on patient survival was obtained by contacting the Resident Service Division of the Ministry of Public Administration and Security, Seoul, Korea.

For patients who died during the study period, the total number and cost of additional biliary drainage procedures after the occlusion of subsequent SEMS or PS (the sum of the costs of ERCP, ERBD, PTBD, PTBD catheter exchange, and stents) were compared between the two groups. The costs were converted from Korean won to U.S. dollars according to annual medical fee schedules and the annual average exchange rate (Table 1)[18-27]. Data were collected until the death of the patient or June 30, 2009. This study was approved by the institutional review boards of the institutions.

Subsequent stent insertion

A diagnosis of SEMS occlusion was made when a patient who had undergone ERBD with a SEMS presented with cholangitis (fever, tenderness in the right upper quadrant or epigastrium, and/or a $\geqslant$ two-fold increase in the serum bilirubin level above the baseline after initial SEMS insertion), or when the total serum bilirubin level was increased $\geqslant$ twofold above the baseline after initial SEMS insertion, even without symptoms or signs of cholangitis. After the diagnosis of SEMS occlusion was made, all patients fit for ERBD underwent the procedure.

ERCP was performed to characterize the cause of the SEMS occlusion using standard- or large-channel duodenoscopes (TJF-240, JF-240, TJF-200, JF-200; Olympus Optical Co., Ltd., Tokyo, Japan). Once stent occlusion was diagnosed, mechanical cleaning with a balloon catheter or a stone extraction basket was usually performed to examine the causes of stent malfunction if the cause was not clear. A guidewire was passed through the stricture, and the stricture length was measured with Tandem RX cannulas (Boston Scientific, Natick, Mass) or Tracer Metro guidewires (Cook Medical, Winston-Salem, NC). After the guidewire had passed through the occluded SEMS, another SEMS or PS was placed through the initial SEMS. Proper placement of the stent was confirmed by fluoroscopy.

In the SEMS group, covered or uncovered Wallstents (Boston Scientific) or uncovered Niti-S stents (Taewoong Medical Inc., Gimpo-si, Korea) were used. In the PS group, 10F Percuflex Amsterdam biliary stents (Boston Scientific), or Cotton-Leung stents (Cook Medical) were used.

Definition of events

Successful subsequent ERBD was defined as placement of the stent across the occluded initial SEMS confirmed by the appropriate radiographic positioning, immediate biliary decompression, and at least a 30% reduction in, or normalization of, the serum bilirubin level ($\leqslant$ 1.2 mg/dL).

Occlusion of subsequent ERBD was diagnosed when the patient developed symptoms or signs of stent occlusion, as described above. Time to stent occlusion was defined as the time between stent insertion and stent occlusion; the overall stent patency was defined as the time between stent insertion and stent occlusion or death of the patient.

Statistical analysis

Qualitative variables were compared using the χ^2 test or Fisher's exact test, where appropriate. The *t* test was used for comparison of quantitative variables. Time to stent occlusion, overall stent patency, and patient survival after the subsequent ERBD were estimated using the Kaplan-Meier method and compared with the log rank test. Factors influencing the time to subsequent stent occlusion were determined using the log-rank test. Two-sided *P*

Table 1 Costs of procedures and stents in US dollars[1]

	Year									
	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
ERCP	56.45	53.69	53.62	58.14	62.18	71.57	79.48	83.61	76.89	71.45
ERBD	175.48	153.70	153.50	166.46	178.02	204.88	227.50	239.33	204.24	178.73
PTBD	91.66	81.60	81.48	88.37	94.51	108.77	120.77	127.06	150.52	168.09
PTBD tube change	26.65	34.61	34.57	37.49	40.09	46.14	51.23	53.90	67.71	77.82
SEMS	697.80	611.19	628.53	661.92	689.23	770.21	825.67	810.86	688.32	641.93
PS	60.47	52.96	54.46	57.36	59.72	66.74	71.55	74.02	59.55	55.54

[1]Costs converted from Korean won to US dollars according to annual medical fee schedules and the annual average exchange rate. ERCP: Endoscopic retrograde cholangiopancreatography; ERBD: Endoscopic retrograde biliary drainage; PTBD: Percutaneous transhepatic biliary drainage; SEMS: Self-expandable metal stent; PS: Plastic stent.

Table 2 Patient characteristics

	SEMS group	PS group	*P* value
No. of patients	29	27	
Gender (M:F)	18:11	19:8	0.512
Age at initial SEMS insertion, median (yr)	66	66	0.352
Diagnoses			0.277
Pancreatic cancer	15	6	
Common bile duct cancer	3	5	
Hilar cholangiocarcinoma	4	4	
Gallbladder cancer	3	6	
Metastatic lymph nodes	3	2	
Ampulla of Vater cancer	1	3	
Hepatocellular carcinoma	0	1	
Anti-cancer therapy	21	15	0.188

SEMS: Self-expandable metal stent; PS: Plastic stent.

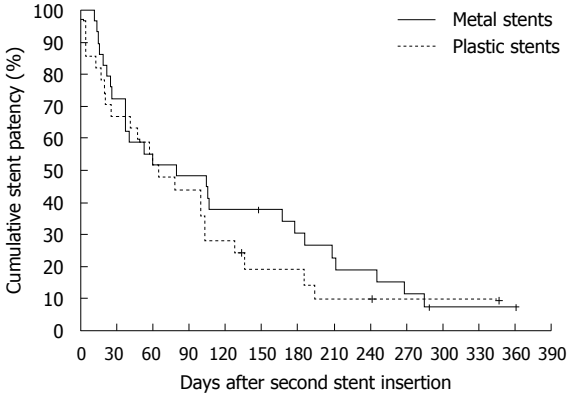

Figure 1 Kaplan-Meier estimation of patency rates of subsequent endoscopic retrograde biliary drainage. There was no significant difference in the patency between self-expandable metal stent and plastic stent ($P = 0.379$).

values of < 0.05 were considered significant. All analyses were performed using SPSS for Windows Ver. 11.0 (SPSS Inc., Chicago, IL).

RESULTS

Patient characteristics

A total of 56 (37 male) patients were evaluated. The median age at initial SEMS insertion was 66 years (range, 38-87 years). The diagnoses were: pancreatic cancer ($n = 21$), gallbladder cancer ($n = 9$), common bile duct cancer ($n = 8$), hilar cholangiocarcinoma ($n = 8$), metastatic lymph nodes ($n = 5$), ampulla of Vater cancer ($n = 4$), and hepatocellular carcinoma ($n = 1$). Thirty-two patients had no biliary drainage procedures prior to the initial SEMS insertion. Initial SEMSs were inserted endoscopically in 46 patients and percutaneously in 10. The median time to occlusion of the first SEMS was 124 d (range, 22-755 d). The causes of the first SEMS occlusion were: tumor ingrowth ($n = 37$), sludge and clogging ($n = 9$), combined ingrowth and sludge ($n = 7$), migration ($n = 2$), and tumor overgrowth ($n = 1$). After occlusion of the initial SEMS, subsequent ERBD with SEMS was performed in 29 patients (uncovered SEMS in 19 and covered SEMS in 10) and PS in 27. Changes in stent selection trends were evident over time. Between 2001 and 2006, 10 SEMSs and 21 PSs were inserted during the subsequent ERBD, whereas between 2007 and 2008, 19 SEMSs and 6 PSs were inserted ($P = 0.001$). No significant difference in the diagnoses of the two stent groups was noted ($P = 0.277$). Twenty-one patients (72.4%) in the SEMS group and 15 (55.6%) in the PS group underwent anti-cancer therapy ($P = 0.188$) (Table 2).

Subsequent ERBD

Stent occlusion after subsequent ERBD occurred after a median of 186 d (range, 11-285 d) in 16 patients (55.2%) in the SEMS group and after a median of 101 d (range, 2-194 d) in 18 patients (66.7%) in the PS group ($P = 0.118$ for time to stent occlusion, $P = 0.379$ for stent occlusion rate). The causes of stent occlusion were tumor ingrowth ($n = 17$), sludge and clogging ($n = 10$), migration ($n = 2$), tumor overgrowth ($n = 2$), duodenal obstruction ($n = 2$), and combined ingrowth and overgrowth ($n = 1$). Overall stent patency was 79 d for the SEMS group and 66 d for the PS group ($P = 0.379$). The cumulative stent patency estimated by the Kaplan-Meier method was 72.4%, 55.2%, 48.3%, and 37.9% for the SEMS group and 66.7%, 55.6%, 40.7%, and 25.9% for the PS group at 30, 60, 90, and 120 d, respectively (Figure 1). There was no correlation between the patency of the first SEMS and subsequent stent that had occluded (correlation coefficient, 0.251, $P = 0.152$).

When adjusted for anti-cancer treatment, the median

Table 3 Comparison of self-expandable metal stent and plastic stent in subsequent endoscopic retrograde biliary drainage (mean ± SD) *n* (%)

	SEMS group (*n* = 29)	PS group (*n* = 27)	*P* value
Follow-up period, median (d)	200	133	0.993
Stent occlusion	16 (55.2)	18 (66.7)	0.379
Time to stent occlusion, median (d)[1]	186	101	0.118
Overall stent patency, median (d)[1,2]	79	66	0.379
No. of additional biliary drainage procedures	2.54 ± 4.12	1.85 ± 1.95	0.457
Total cost of subsequent biliary drainage procedures ($)	410.04 ± 692.60	630.16 ± 671.63	0.260
Survival after subsequent ERBD, median (d)[1]	200	133	0.225

[1]Results of log-rank test; [2]Period between stent insertion and stent occlusion or death of the patient. SEMS: Self-expandable metal stent; PS: Plastic stent; ERBD: Endoscopic retrograde biliary drainage.

time to stent occlusion was not significantly different between the SEMS and PS groups. For the patients who underwent anti-cancer therapy, the median time to stent occlusion was 177 d for the SEMS group and 104 d for the PS group; in patients who did not undergo anti-cancer treatment, the median time to stent occlusion was 186 d for the SEMS group and 79 d for the PS group ($P = 0.120$).

When covered SEMS group ($n = 10$) and uncovered SEMS group ($n = 19$) were compared, the median time to stent occlusion was 208 d (range, 22-268 d) for covered SEMS and 106 d (range, 37-285 d) for uncovered SEMS ($P = 0.659$). The median overall stent patency was 186 d (range, 19-268 d) for the covered SEMS and 60 d (range, 11-285 d) for uncovered SEMS ($P = 0.599$).

Fifty patients died during the study period. The mean number of additional biliary drainage procedures after subsequent ERBD in patients who died during the study period was 2.54 ± 4.12 for the SEMS group and 1.85 ± 1.95 for the PS group ($P = 0.457$). The mean total cost of additional biliary drainage procedures after the occlusion of subsequent SEMS or PS was $410.04 ± 692.60 for the SEMS group and $630.16 ± 671.63 for the PS group ($P = 0.260$). There was no difference in the median follow-up period (200 d for SEMS group *vs* 133 d for PS group, $P = 0.993$). The median survival was not significantly different between the two groups (200 d for SEMS group *vs* 133 d for PS group, $P = 0.225$) (Table 3).

Factors influencing the patency of the subsequent stent, for both SEMSs and PSs, were analyzed. Tumor ingrowth as the cause of the initial SEMS occlusion was the only factor associated with a shorter median time to subsequent stent occlusion (101 d for patients with tumor ingrowth *vs* 268 d for patients without tumor ingrowth, $P = 0.008$). Gender, age at initial SEMS insertion, diagnosis (pancreatic cancer *vs* non-pancreatic cancer), biliary drainage prior to initial SEMS insertion, anti-cancer therapy, and presentation at initial SEMS occlusion with cholangitis had no impact on the subsequent stent patency (Table 4).

Table 4 Univariate analysis of factors associated with time to second stent occlusion

Factor	Time to second stent occlusion, median (d)	*P* value
Sex		0.273
Male (*n* = 37)	103	
Female (*n* = 19)	137	
Age at initial SEMS insertion (yr)		0.697
⩾ 65 (*n* = 31)	137	
< 65 (*n* = 25)	104	
Diagnosis		0.363
Pancreatic cancer (*n* = 21)	137	
Others (*n* = 35)	103	
Biliary drainage prior to initial SEMS insertion		0.924
Yes (*n* = 24)	137	
No (*n* = 32)	104	
Cause of initial SEMS occlusion		0.008
Ingrowth (*n* = 44)	101	
Not ingrowth (*n* = 12)	268	
Anti-cancer therapy		0.444
Yes (*n* = 36)	137	
No (*n* = 20)	103	
Cholangitis on initial SEMS occlusion		0.244
Yes (*n* = 33)	103	
No (*n* = 23)	186	
Covered initial SEMS		0.918
Yes (*n* = 4)	186	
No (*n* = 52)	104	

Results of log-rank test. SEMS: Self-expandable metal stent.

Cox regression analysis demonstrated that tumor ingrowth was associated with shorter time to subsequent stent occlusion (hazard ratio, 8.45; 95% confidence interval, 2.44-29.29; $P = 0.001$).

DISCUSSION

In this retrospective study of the endoscopic management of occluded SEMS for unresectable malignant biliary obstruction, no significant difference was observed for the patency or time to stent occlusion between the PS and the SEMS. In addition, no differences were observed with regard to the number or cost of additional biliary drainage procedures and patient survival. Tumor ingrowth as the cause of the initial SEMS occlusion was the only factor associated with a shorter time to subsequent stent occlusion.

There have been a few retrospective studies regarding the management of occluded SEMSs, with variable results. The study reported by Tham *et al*[15] analyzed 38 patients with 44 Wallstent occlusions. Wallstent occlusion was managed by insertion of another Wallstent in 19, insertion of a PS in 20, and mechanical cleaning in 5 cases. No significant difference in the duration of overall stent patency among the three groups was observed. Another report by Bueno *et al*[14] analyzed 34 patients with Wallstent occlusions. Six patients underwent mechanical cleaning, 4 had placement of a second Wallstent, and 24 had a PS insertion. The median duration of stent patency was 192 d for the second Wallstent, 90 d for the PS, and 21 d for the mechanical cleaning. Although the second Wall-

stent showed a significantly longer duration of patency, this study was limited by the relatively small number of patients who underwent second Wallstent insertion.

Two studies regarding this subject were published in 2008. Togawa *et al*[16] evaluated 40 patients with occluded uncovered SEMSs. Covered SEMSs were inserted in 26 patients, uncovered SEMSs in 7, and PSs in 7. The mean overall patency of the subsequent ERBD was 141.3, 219.6, and 57.9 d for uncovered SEMS, covered SEMS, and PS, respectively. It should be noted that the majority of second SEMSs used in this study were Diamond stents. Rogart *et al*[17] reported that in their experience, placing a second SEMS provided the lowest reocclusion rate and the longest time to reintervention. However, the total number of patients in their study was 27, with a second SEMS insertion in 14, PS insertion in 11, and mechanical cleaning in 2.

Since SEMS and PS did not show significant differences in the time to stent occlusion in our study, the factors influencing the time to subsequent stent occlusion in both groups were assessed. Tumor ingrowth as the cause of initial SEMS occlusion was the only factor associated with shorter time to second stent occlusion, regardless of the material used for the second stent. Tumor ingrowth was the most common cause of the initial SEMS occlusion in this study. Unlike other causes of SEMS occlusion such as tumor overgrowth, sludge or migration, ingrowth may be difficult to overcome by subsequent stent insertion because a long segment of the bile duct might be involved with more compressive force on the stent, thus interfering with the expansion of the SEMS. Moreover, the initial SEMS embedded in the tumor tissue may serve as a rigid framework, adding more resistance to the axial force of the subsequent SEMS. If this was the case, a PS with its fixed diameter may not be inferior to SEMS in subsequent ERBD. A covered SEMS or SEMS with a high radial force may be useful in this setting. Previous reports have shown variable results on this issue. Togawa *et al*[16] demonstrated that covered SEMS were superior to uncovered SEMS for the management of occluded SEMSs. However, the study by Rogart *et al*[17] failed to show that the covered SEMS was superior. A prospective study comparing the efficacy of such SEMSs in the management of initial SEMS occlusion is needed.

The limitations of this study include the following. This was a non-randomized, retrospective study which may result in selection bias. No predefined follow-up protocol was available. Patients with less than 8 wk of follow-up without stent occlusion or death were excluded, since most, if not all, of these patients were lost to follow-up right after the subsequent ERBD. However, this study includes the largest number of patients with SEMS occlusion to date.

In conclusion, subsequent ERBD with PS had similar patency, and number and cost of additional biliary drainage procedures, compared to the SEMS in the endoscopic management of occluded SEMS. Tumor ingrowth as the cause of initial SEMS was the only factor associated with shorter time to second stent occlusion. Therefore, strategies to overcome tumor ingrowth during subsequent ERBD might be beneficial to this subset of patients.

COMMENTS

Background

There are limited data regarding the management of occluded self-expanding metal stents (SEMSs) in palliation of unresectable malignant biliary obstruction.

Innovations and breakthroughs

This study identified tumor ingrowth as the factor associated with shorter time to second stent occlusion.

Applications

In the endoscopic management of occluded SEMS, subsequent endoscopic retrograde biliary drainage with a plastic stent had similar patency and number of additional biliary drainage procedures compared to the SEMS.

Peer review

This is an interesting study in an area without high grade evidence. The authors acknowledge the limitations of their methodology (non-randomized, retrospective).

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BRIEF ARTICLE

Predictive factors associated with malignancy of intraductal papillary mucinous pancreatic neoplasms

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Abstract

AIM: To identify preoperative predictive factors associated with malignancy of intraductal papillary mucinous neoplasms (IPMNs) of the pancreas.

METHODS: Between April 1995 and April 2010, 129 patients underwent surgical resection for IPMNs at our institute and had confirmed pathologic diagnoses. The medical records were retrospectively reviewed and immunohistochemical staining for mucin (MUC) in pancreatic tissues was performed.

RESULTS: Univariate analysis showed that the following five variables were closely associated with malignant IPMNs preoperatively: absence of extrapancreatic malignancy; symptoms; tumor size > 4 cm; main pancreatic duct (MPD) size > 7 mm; and lymph node enlargement on preoperative computed tomography (CT). Multivariate analysis revealed that the following two factors were significantly associated with malignant IPMNs preoperatively: MPD size > 7 mm [odds ratio (OR) = 2.50]; and lymph node enlargement on preoperative CT (OR = 3.57). No significant differences in the expression of MUC1, MUC2 and MUC5AC were observed between benign and malignant IPMNs.

CONCLUSION: MPD size > 7 mm and preoperative lymph node enlargement on CT are useful predictive factors associated with malignancy of IPMNs.

Key words: Intraductal papillary mucinous neoplasms; Malignancy; Predictive factors; Pancreatic neoplasms

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INTRODUCTION

Intraductal papillary mucinous neoplasms (IPMNs) of the pancreas are characterized by intraductal proliferation of neoplastic mucinous cells, which usually form papillae, and cystic dilation of the pancreatic ducts, thus forming

a clinically and macroscopically detectable mass[1]. The classification of IPMNs according to the World Health Organization nomenclature[1] is as follows: adenoma; borderline tumor; carcinoma *in situ* (CIS); and invasive carcinoma. Depending on the degree of dysplasia, treatment of IPMNs includes conservative treatment and radical pancreatectomy with extended resection. Therefore, it is important to diagnose the grade of IPMNs preoperatively.

Previous studies have revealed predictive factors of invasiveness or malignancy of IPMNs of the pancreas[2-8]. Factors, such as age at the time of diagnosis, tumor size, main pancreatic duct (MPD) size, duct type, the presence of mural nodules, the presence of symptoms, and thick septum are associated with invasiveness or malignancy of IPMNs. However, the results of predictive factors have not been consistent with each other and some are not diagnostic. The pre-operative differential diagnosis between benign and malignant IPMNs, or between non-invasive and invasive IPMNs is not easy, despite the development of diagnostic modalities.

Recently, several studies have demonstrated the expression of mucin (MUC) on pancreatic tumors by immunohistochemical staining[9-13]. MUC1 (membrane mucin) is related to the invasive proliferation of tumors, while the expression of MUC2 (intestinal-type secretory mucin) is related to noninvasive proliferation of tumors[14].

The purpose of the current study was to identify preoperative predictive factors associated with malignancy of IPMNs of the pancreas by reviewing patients' records, and to reveal the role of MUC expression in differentiating malignant IPMNs using several specific antibodies.

MATERIALS AND METHODS

Patients and clinical characteristics

Between April 1995 and April 2010, 129 patients who underwent surgical resection for IPMNs of the pancreas at the Samsung Medical Center in Seoul, Korea, and had a confirmed pathological diagnosis were included. The medical records were retrospectively reviewed to obtain the demographic characteristics.

We analyzed variable factors, such as age at the time of diagnosis, sex, presence or absence of diabetes mellitus, alcohol intake history, and cigarette smoking. Symptomatic IPMNs were defined as the presence of abdominal pain and/or jaundice. Recently, IPMNs have been shown to be associated with a high incidence of extrapancreatic gastrointestinal neoplasms[15,16], thus, we assessed preoperatively the presence of extrapancreatic gastrointestinal cancers in the study population.

We determined the serum levels of total bilirubin, amylase, lipase, carcinoembryonic antigen (CEA), and carbohydrate antigen (CA) 19-9 within 1 mo preoperatively. All patients underwent preoperative computed tomography (CT). We assessed the duct type, tumor size, location, MPD size, and presence of mural and intra-abdominal lymph node enlargement on CT. Adenomas and borderline tumors were benign tumors, and CIS and invasive carcinomas were malignant tumors.

Immunohistochemical staining

The surgical specimens were fixed with 10% formalin and cut at intervals of 5 mm. The tumor samples were embedded in paraffin, and the histological sections were cut into 5-μm thick slices for hematoxylin and eosin staining. Immunohistochemical staining for p53, MUC1, MUC2 and MUC5AC was performed on the serial sections for IPMN tissues. The 5-μm thick sections were deparaffinized with xylene, and rehydrated in alcohol. After rinsing in PBS, the sections were incubated for 1 h at room temperature with DF3 (1:50 dilution; Toray-Fuji Bionics, Tokyo, Japan) for MUC1 antigen, Ccp58 (undiluted; Biogenex, San Ramon, CA, USA) for MUC2 antigen, and CLH2 (1:50 dilution; Novocastra, Newcastle, UK) for MUC5AC antigen. In addition, sections were incubated for 25 min at room temperature with BP53.12 (1:400 dilution; Zymed, San Francisco, CA, USA) for p53 antigen. The sections were rinsed with tap water. Positivity of the immunohistochemical stain was judged by the presence of staining in the intraductal tumor cells.

Statistical analysis

Continuous data are presented as the mean ± SD or median and range. The χ^2 test or Fisher's exact test was used to evaluate differences between categorical variables. Significant predictors in the univariate analysis were included in the logistic regression model for multivariate analysis. Differences at $P < 0.05$ were considered statistically significant. Statistical analyses were performed using SPSS 17.0k software (SPSS Inc., Chicago, IL, USA).

RESULTS

Patient characteristics

One hundred and twenty-nine patients underwent surgical resection and were confirmed with IPMN of the pancreas (Table 1). The study group included 94 men and 35 women, with a mean age at the time of diagnosis of 60.9 years (range, 32-77 years). More than half of the IPMNs were detected incidentally in asymptomatic patients during routine check-ups. The duct type included the main duct in 56 patients (43.4%), a branch duct in 46 (35.7%), and mixed ducts in 27 (20.9%). Eighty-one of the patients (62.8%) were diagnosed with benign tumors (adenomas and borderline tumors) and 48 (37.8%) had malignant tumors (CIS and invasive carcinomas).

Comparisons of characteristics between benign and malignant IPMNs

There were no differences in age at the time of diagnosis and sex between the patients with benign and malignant IPMNs (Table 2). In both groups, the proportion of current smokers and alcohol drinkers was no different. Of the IPMNs in patients with diabetes mellitus, 37.5% were malignant and 27.1% were benign tumors ($P > 0.05$). Symptomatic IPMNs were observed in 31 of the patients (64.6%) with malignant tumors and 32 (39.5%) with benign tumors ($P = 0.007$). The proportion of patients with tumors > 4 cm in size was greater in patients with malignant IPMNs

Table 1 Demographics and subtypes of intraductal papillary mucinous neoplasms (*n* = 129) (mean ± SD) *n* (%)

Features	Value
Age (yr)	60.9 ± 9.6 (range, 32-77)
Sex (M:F)	94:35
Symptoms	
(-)	66 (51.2)
(+)	63 (48.8)
Mean tumor size (cm)	3.7 ± 3.2 (range, 0.6-16.5)
Mean MPD size (mm)	7.4 ± 4.8 (range, 1.0-30.0)
Tumor location	
Pancreatic head or uncinate process	80 (62.0)
Body or tail	49 (28.0)
Duct types on CT	
Main duct	56 (43.4)
Branch duct	46 (35.7)
Mixed duct	27 (20.9)
Pathologic subtypes	
Adenoma	51 (39.5)
Borderline	30 (23.3)
CIS	5 (3.9)
Invasive	43 (33.3)

MPD: Main pancreatic duct; CT: Computed tomography; CIS: Carcinoma *in situ*.

(37.5%) than benign tumors (18.5%, $P = 0.019$). The MPD was > 7 mm in diameter more frequently in malignant (62.5%) than in benign (33.3%, $P = 0.002$) tumors. According to CT, there were no significant differences in the proportion of patients with mural nodules, the tumor location within the pancreas, and duct types ($P > 0.05$). Intra-abdominal lymphadenopathy, defined as lymph nodes > 1.5 cm in size on preoperative CT, existed in 31.2% of malignant and 9.8% of benign tumors ($P = 0.003$). The mean serum level of amylase in patients with benign IPMNs was higher than in those with malignant tumors (128.0 ± 13.5 U/L *vs* 86.8 ± 61.1 U/L, $P = 0.173$). The mean serum levels of total bilirubin (2.1 ± 4.6 mg/dL), CEA (2.2 ± 1.3 ng/mL), and CA 19-9 (867.9 ± 3958.5 U/mL) in patients with malignant IPMNs was similar to those in patients with benign tumors (0.7 ± 0.4 mg/dL, 6.7 ± 31.9 ng/mL, and 34.5 ± 81.4 U/mL, respectively, $P > 0.05$).

Patients with benign tumors had more extrapancreatic gastrointestinal malignancies before or during the diagnosis of IPMN than patients with malignant tumors (28.1% *vs* 6.2%, $P = 0.014$). Four of 23 patients with IPMNs with extrapancreatic malignancies were women and more than half of all cases were detected at the same time as the diagnosis of IPMN was established. Gastric cancer (10 patients) and lower gastrointestinal tract cancer (eight patients) comprised the majority of extrapancreatic malignancies (Table 3).

Analysis of preoperative findings associated with malignant IPMNs

Univariate analysis showed that the following preoperative variables were closely associated with malignant IPMNs: absence of extrapancreatic malignancy; symptoms; tumor size > 4 cm; MPD size > 7 mm; and lymph node enlargement on preoperative CT (Table 2). Two of five factors that were significant predictors based on univariate analysis were identified as significant on multivariate analysis: MPD size > 7 mm [odds ratio (OR) = 2.50]; and lymph node enlargement on preoperative CT (OR = 3.57, Table 4).

Table 2 Univariate analysis of preoperative findings associated with malignant intraductal papillary mucinous neoplasms of the pancreas

Factor	Benign (*n* = 81)	Malignant (*n* = 48)	*P* value
Age (yr)			
< 60	27	24	0.063
⩾ 60	54	24	
Sex			
Male	64	30	0.064
Female	17	18	
Alcohol			
Non-alcoholic	50	37	0.083
Alcoholic	31	11	
Smoking			
Non-smoker	53	35	0.437
Current smoker	28	13	
Diabetes mellitus			
No	59	30	0.242
Yes	22	18	
Symptoms			
No	49	17	0.007
Yes	32	31	
Tumor size (cm)			
⩽ 4	66	30	0.019
> 4	15	18	
MPD diameter (mm)			
⩽ 7	54	18	0.002
> 7	27	30	
Mural nodules on CT			
No	77	44	0.469
Yes	4	4	
Amylase (U/L)			
⩽ 100	53	37	0.173
> 100	28	11	
Total bilirubin (mg/dL)			
⩽ 1.2	75	41	0.231
> 1.2	6	7	
CEA (ng/mL)			
⩽ 6	79	48	0.529
> 6	2	0	
CA 19-9[1] (U/mL)			
⩽ 37	65	32	0.063
> 37	10	12	
Location in pancreas			
Head	52	28	0.575
Body or tail	29	20	
Intra-abdominal lymphadenopathy on CT			
No	73	33	0.003
Yes	8	15	
Duct type			
Main or mixed	47	36	0.059
Branch duct	34	12	
Extrapancreatic malignancy			
No	61	45	0.014
Yes	20	3	

[1]Missing data on CA 19-9 in some patients was presented. MPD: Main pancreatic duct; CT: Computed tomography; CEA: Carcinoembryonic antigen; CA: Carbohydrate antigen.

Table 3 Characteristics of intraductal papillary mucinous neoplasms with extrapancreatic malignancies

No.	Sex	Age (yr)	Diagnosis	Extrapancreatic malignancy	Time of occurrence
1	M	62	Adenoma	Early gastric cancer	Syn
2	M	55	Adenoma	Early gastric cancer	Syn
3	M	66	Adenoma	Colon tubular adenoma	Syn
4	M	58	Borderline	Advanced gastric cancer	Prior
5	F	54	Adenoma	Advanced gastric cancer	Syn
6	F	72	Adenoma	Advanced gastric cancer	Syn
7	F	70	Adenoma	Appendix mucinous tumor	Syn
8	M	64	Adenoma	Colon cancer	Prior
9	M	72	Borderline	Colon cancer	Prior
10	M	56	Invasive	Colon cancer	Syn
11	M	59	Adenoma	Early gastric cancer	Syn
12	M	48	Borderline	Early gastric cancer	Prior
13	M	60	Adenoma	Early gastric cancer	Prior
14	M	67	Adenoma	Early gastric cancer	Syn
15	M	62	Borderline	Early gastric cancer	Syn
16	M	53	Adenoma	Gastric SMT	Prior
17	M	66	CIS	Prostate cancer	Prior
18	M	68	Adenoma	Renal cell carcinoma	Syn
19	M	64	Adenoma	Rectal cancer	Prior
20	M	74	Adenoma	Rectal cancer	Prior
21	M	76	Adenoma	Rectal cancer	Prior
22	F	60	Adenoma	Rectal cancer	Syn
23	M	61	Adenoma	Rectal carcinoid tumor	Syn

SMT: Submucosal tumor; CIS: Carcinoma *in situ*; Prior: Prior to intraductal papillary mucinous neoplasm (IPMN); Syn: Synchronous to IPMN.

Immunohistochemical expression of malignant IPMNs

The expression of pancreatic tissues was present in 22.2%, 29.6%, 96.3% and 11.1% of benign IPMNs for MUC1, MUC2, MUC5AC and p53, respectively (Table 5). In patients with malignant IPMNs, the expression of mucin genes was 17.6%, 57.1%, 94.1%, and 34.5% with immunohistochemical staining for MUC1, MUC2, MUC5A, and p53, respectively. No significant differences in expression of MUCs were observed according to the degree of dysplasia.

DISCUSSION

Several studies regarding the natural course of IPMNs have shown that tumor size < 3 cm, branch duct type, and no mural nodules are low-risk factors of malignancy[17]; thus, the pancreas can be conserved until the tumor progresses to an invasive carcinoma. Therefore, a therapeutic strategy for IPMNs should be based on the stage of the malignancy.

Previous studies that have investigated predictive factors for malignant or invasive IPMNs preoperatively were based on univariate analysis, with only four studies being performed using multivariate analysis[3,5-7]. Our data showed that the presence of extrapancreatic malignancies, symptoms, tumor size > 4 cm, MPD dilatation > 7 mm, and intra-abdominal lymphadenopathy on preoperative CT were significant predictive factors of malignant IPMNs, based on univariate analysis, and only two of the five factors (MPD > 7 mm and intra-abdominal lymphadenopathy on CT) were statistically significant on multivariate analysis. The present study is believed to be the first to report intra-abdominal lymphadenopathy on CT as a predictive factor, which is not well known for sensitivity and specificity. According to our study, we emphasized the diagnostic importance of CT and recommend it as a follow-up tool to predict malignant changes.

Table 4 Multivariate analysis of preoperative findings associated with malignant intraductal papillary mucinous neoplasms

Factors	OR	95% CI	*P* value
Extrapancreatic malignancy - none	3.58	0.95-13.51	0.059
Symptomatic	1.89	0.83-4.32	0.127
Tumor size > 4 cm	2.35	0.96-5.76	0.062
MPD diameter > 7 mm	2.50	1.10-5.65	0.028
Preoperative CT lymphadenopathy	3.57	1.22-10.37	0.020

MPD: Main pancreatic duct; CT: Computed tomography; OR: Odds ratio; CI: Confidence interval.

Table 5 Immunohistochemical staining of intraductal papillary mucinous neoplasms *n* (%)

Markers	Benign IPMN	Malignant IPMN
MUC1	2/9 (22.2)	3/15 (17.6)
MUC2	8/27 (29.6)	8/14 (57.1)
MUC5AC	26/27 (96.3)	16/17 (94.1)
p53	2/18 (11.1)	10/29 (34.5)

IPMN: Intraductal papillary mucinous neoplasm; MUC: Mucin.

Symptomatic tumors > 3 cm in size are known as important factors for malignancy of branch duct type IPMNs according to international guidelines[18]. In another study[5], the optimal cut-off value for IPMN size in the detection of malignancies was 4 cm. The analysis of our patients divided by tumor size of 3 cm was not statistically significant, thus, we set the cut-off value at 4 cm, which was significantly associated with malignant IPMNs.

A number of previous studies have reported that malignant or invasive IPMNs are more frequently observed in those who have main duct or mixed duct type than branch duct type IPMN[5,18,19]. We showed that the duct type of IPMNs was not affected by malignant transformation of tumors. Patients with the branch duct type in our institute usually underwent wait and watch management, therefore, they were not included with the study group who underwent surgical resection. For the accuracy of predictive factors, follow-up data of branch duct type IPMNs will be necessary in future studies.

The presence of mural nodules has also been reported as a predictive factor of malignant IPMNs[4-7], but it was not associated with malignant tumors in the current study. Mural nodules are usually detected on preoperative multidetector row CT. Mucinous secretions in cystic masses appear to be misdiagnosed as mural nodules because of similarities on CT imaging. Therefore, mural nodules are not diagnostic predictive factors associated with malignant IPMNs.

Our study revealed that benign IPMNs were more likely to have extrapancreatic gastrointestinal malignan-

cies than malignant tumors, which is in agreement with other studies[11,20-22]. The basis for this result has not been explained, but the hypothesis has been advanced that patients with extrapancreatic malignancies were excluded from the study group due to death from underlying cancers[21]. In our study, 28 of 129 patients had an extrapancreatic malignancy and many cases were detected during preoperative evaluation of IPMNs. Gastric or lower gastrointestinal cancers comprised most of the extrapancreatic malignancies, therefore, upper endoscopy and colonoscopy are necessary to detect associations with other gastrointestinal malignancies at the time diagnosis of IPMNs.

IPMN cells secrete a thick MUC that causes dilatation of the MPD. The dysregulation of one or more types of MUCs could lead to formation of malignant IPMNs. There have been several studies[9-14] regarding the expression of MUC in pancreatic tumors. For example, MUC1 is known to be a marker of invasive carcinoma and MUC2 is found only in the intestinal type of IPMNs[10]. In the current study, a difference in the expression of MUC on benign and malignant IPMNs was not demonstrated. Positive staining of MUC1 was shown in 22.2% of adenomas or borderline IPMNs and MUC5AC expression was shown in > 94% of all IPMNs. Cell cycle modulator molecules, for example p53, were similar in both groups.

This study was limited in that it was retrospective and evaluated the predictive factors associated with malignancy, which were diagnosed in most cases by CT. Another limitation was that the examination of MUC expression according to pathological subtypes[23], such as gastric, intestinal, pancreatobiliary, and oncocytic, was not performed, and the study group consisted only of patients who underwent surgical resection. We do not know the significance of patients who received conservative treatment or adjuvant chemoradiation therapy.

In conclusion, a MPD size > 7 mm and preoperative intra-abdominal lymphadenopathy on CT are useful predictive factors associated with malignancy of IPMNs. The proper diagnosis of malignancies in patients with IPMNs is needed for those who are undergoing surgical resection.

COMMENTS

Background

Several investigators have revealed predictive factors of invasiveness of malignancy of intraductal papillary mucinous neoplasm (IPMN), but the results of predictive factors have not been consistent with each other and some are not diagnostic.

Research frontiers

Several recent studies have investigated the role of mucin expression to differentiate the malignancy of IPMN, by using several specific antibodies. Also, it is known that IPMN is associated with a high incidence of extrapancreatic neoplasms.

Applications

It appears to be important to determine the surgical resection of IPMN according to malignant potential.

Terminology

The main pancreatic duct type of IPMNs was treated with surgical resection due to its malignancy, but the branch duct type was sometimes treated without surgery because most tumors of this type of tumor have benign characteristics.

Peer review

This is a good retrospective study. Whether or not the results will prove useful in a prospective fashion is appropriately addressed by the authors.

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BRIEF ARTICLE

Specific intronic *p53* mutation in esophageal squamous cell carcinoma in Southern Thailand

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Abstract

AIM: To investigate *p53* mutations in esophageal cancer in a high-risk population, and correlate them with smoking, alcohol consumption and betel chewing.

METHODS: One hundred and sixty-five tumor samples of esophageal squamous cell carcinoma (ESCC) obtained from a university hospital in Songkhla province, Southern Thailand were investigated for *p53* mutations in exons 5-8, using polymerase chain reaction-single strand conformation polymorphism analysis, followed by direct sequencing. A polymerase chain reaction-restriction fragment length polymorphism (RFLP) assay was additionally used to confirm possible germline mutation in intron 6. A history of risk habits was obtained by interviews. The association between risk habits and mutation frequency was evaluated using the χ^2 test.

RESULTS: The studied specimens were from 139 male and 26 female patients with ESCC, treated at Songklanagarind Hospital. Most of the patients were smokers (86.7%) and alcohol consumers (72.73%), and 38.3% were betel chewers. Forty-three mutations of the *p53* gene were detected in 25.5% (42/165) of tumor samples. Mutations were most commonly found in exon 5 (25.6%) and exon 8 (25.6%). Mutations in the hot-spot codon 248 were found in four cases (9.3% of all mutations). G:C→C:G (30.23%), G:C→A:T (27.90%) and G:C →T:A (16.28%) were the prevalent spectra of mutations. Unexpectedly, among 10 intronic mutations, eight cases harbored a similar mutation: G→C substitution in intron 6 (nucleotide 12759, GenBank NC_000017). These were additionally confirmed by the RFLP technique. Similar mutations were also detected in their matched blood samples using RFLP and direct sequencing, which suggested germline mutations. There was no significant correlation between risk habits and *p53* mutation frequency.

CONCLUSION: A proportion of Thai ESCC patients harbored specific intronic *p53* mutations, which might be germline mutations. Further studies are needed to explore this novel finding.

Key words: Esophageal cancer; Squamous cell carcinoma; *p53* gene; Germline mutation; Mutation; Intron

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INTRODUCTION

Esophageal cancer is the eighth most common cancer worldwide, and there were 462 000 new cases in 2002[1]. It is a disease of high mortality, and ranks as the sixth most common cause of cancer death. There is a marked variation in incidence in different regions of the world; a 20-fold difference is observed between high-risk China and low-risk Western Africa. Other areas of moderately high risk are Southern and Eastern Africa, South-Central Asia, and Japan[1]. It seems that environmental carcinogens are responsible for these geographic differences and the different histological types. Tobacco and alcohol use are the main risk factors in Europe and North America[2,3], and other factors including betel chewing, hot beverages, fermented food, nutritional deficiencies or familial predisposition can be responsible for high rates in other high- or moderate-risk regions[4-7].

The incidence of esophageal cancer in Thailand is relatively low when one considers the country-wide estimates, with an age-standardized incidence rate (ASR) of 4.7 per 100 000 males in 1999[8]. However, the incidence is exceptionally high in Songkhla province in Southern Thailand, with an ASR of 8.1 per 100 000 males, which is close to worldwide incidence. In this region, oral cancer is event more prevalent, with the highest incidence (ASR 9.4 per 100 000 males) compared to other regions of the country. Most esophageal cancer cases in Thailand are squamous cell carcinomas. In our previous case-control study, alcohol consumption, cigarette smoking and betel quid chewing were found to be strong risk factors for esophageal squamous cell carcinoma (ESCC)[9].

The *p53* tumor suppressor gene is an important gene in cell cycle regulation and apoptosis. Mutations in the *p53* gene have been implicated as crucial events in the development of various cancers, including ESCC[10], and they have been identified as a vulnerable target for critical DNA damage. Analysis of *p53* mutations in various human cancers has denoted a characteristic mutational pattern that is related to specific endogenous as well as exogenous carcinogen-related agents; a finding that has given rise to the term "mutagen fingerprints" in DNA[11].

p53 mutations in ESCC from Thailand have been reported by two groups in 1997 and 2000[12,13]. However, the numbers of cases were small and the relationship between mutations and risk habits were not explicitly evaluated. Here, we analyzed the *p53* mutation profile of a larger sample set (165 cases) of ESCC using single-strand conformation polymorphism (SSCP) analysis and direct sequencing. In addition, the relationship between mutation frequency and risk habits, namely alcohol consumption, cigarette smoking and betel quid chewing, was examined.

MATERIALS AND METHODS

Patients and samples

Patients who were diagnosed with ESCC and treated at Songklanagarind Hospital during 1999-2005 were considered as candidates for the study. The study was approved by the Ethics Committee of the Faculty of Medicine, Prince of Songkla University, and informed consent was obtained from the patients. Data concerning detailed histories of tobacco use, alcohol consumption and betel chewing were obtained *via* face-to-face interviews using structured questionnaires. Only cases with available fresh-frozen tissue samples were included. Tissue samples were obtained from biopsy or surgical resection specimens, snapped frozen and stored at -80℃ until DNA extraction. All of the cases were primary tumors that had not been treated with radiation or chemotherapy.

Polymerase chain reaction-SSCP analysis

DNA was extracted from frozen tissues by standard methods. The tissue was digested overnight at 37℃ in lysis buffer that contained 10% SDS, 10 mmol/L Tris, pH 8.0, 10 mmol/L NaCl, 10 mmol/L EDTA and 20 μL 10 mg/mL proteinase K. DNA extraction was performed using the phenol-chloroform method and it was precipitated by 1/10 volume of 4.0 mol/L NaCl and two volumes of cold absolute ethanol.

Exons 5-8 of the *p53* gene were polymerase chain reaction (PCR)-amplified from tumor DNA, and mutations were detected by SSCP analysis. Samples that showed band shift were subjected to direct sequencing. Four sets of primer used were as follows: exon 5: TCTTCCTACAGTACTCCCCT sense, AGCTGCTCACCATCGCTATC antisense; exon 6: GATTGCTCTTAGGTCTGGCC sense, GCAAACCAGACCTCAGGCGG antisense; exon 7: TTATCTCCTAGGTTGGCTCT sense, GCTCCTGACCTGGAGTCTTC antisense; exon 8: TCCTGAGTAGTGGTAATCTA sense, GCTTGCTTACCTCGCTTAGT antisense.

PCR reactions were performed in a 50-μL volume reaction mixture that contained 0.5 μg genomic DNA, 10 pmol each primer, 100 mmol/L Tris, pH 8.3, 500 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, and 1.25 U AmpliTag Gold (Perkin-Elmer, Foster City, CA, USA). Amplification was carried out in a Perkin-Elmer 480 DNA Thermal Cycler. The PCR conditions were 95℃ for 10 min, followed by 35 cycles of 94℃ denaturation for 1 min, 58℃ annealing for 1 min, and 72℃ extension for 1 min. The final extension was conducted at 72℃ for 10 min.

For SSCP analysis, 2 μL PCR product was mixed with 5 μL 95% deionized solution that contained 0.1% bromophenol blue. The mixture was heat-denatured at 100℃ for 5 min and rapidly placed on ice. Four microliters of each denatured product of exons 5 and 8 were loaded on to the 12% polyacrylamide gel (10 cm × 8 cm × 0.75 cm) with 5.26% crosslinking (19:1 acrylamide/bisacrylamide), supplemented with 5% glycerol. For exons 6 and 7, a ratio of 49:1 acrylamide/bisacrylamide (2.04% crosslinking) was used. Electrophoresis was performed in an ice box (12℃) at 2 W and constant 10 mA for 5 h for exons 5 and 8 and 1 h for exons 6 and 7. Positive controls, which consisted of samples that had been confirmed by direct sequencing to contain the *p53* mutation, were run with each SSCP gel that was stained with silver nitrate. All positive cases were confirmed at least once by a separated PCR reaction and SSCP run.

DNA sequencing

The PCR products that showed band shift on the SSCP gel were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and then directly sequenced using the Ready Reaction Dye Terminator Cycle Sequencing kit (Perkin-Elmer). The primers used in sequencing were the same as those used in the PCR. Sequencing was performed on an automated sequencer (ABI-Prism 310, Applied Biosystems, Foster City, CA, USA). All mutations were confirmed by sequencing both DNA strands.

For intron 6 mutation at nucleotide 12759, other primers, not overlapped to the mutation point, were used (forward 5'-GCCTCTGATTCCTCACTGAT-3'; reverse 5'-TAAGCAGCAGGAGAAAGCCCC-3'). This experiment was also performed on four available matched blood samples and the sequencing was performed on an automated sequencer (ABI-Prism 3130).

PCR-restriction fragment length polymorphism analysis to detect intronic G→C at nucleotide 12759

As a significant number of cases showed G→C substitution in intron 6 at the 18th base after the end of exon 6 (corresponding to nucleotide 12759 based on GenBank NC_000017), we additionally confirmed this mutation through restriction fragment length polymorphism (RFLP) analysis. This analysis was also performed on matched blood samples of these cases to investigate whether they were germline mutations.

The 181-bp PCR product was amplified using primers, forward 5'-GCCTCTGATTCCTCACTGAT-3'; and reverse 5'-TTAACCCCTCCTCCCAGAGA-3'. The PCR was performed with 100 ng genomic DNA that contained 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 2.0 mmol/L $MgCl_2$, 37.5 μmol/L each nucleotide, and 1.25 U Taq polymerase. The cycling conditions were 95℃ for 5 min, followed by 35 cycles of 95℃ denaturation for 1 min, 60℃ annealing for 1 min, and 72℃ extension for 1 min, with a final extension of 72℃ for 10 min. A 10-μL aliquot of each successful reaction was digested with 10 U *Bsa*HI restriction enzyme (New England Biolabs, Beverly, MA, USA) in 2.5 μL 10 × NEB4 buffer with 12.5 μL water at 37℃ for 2 h. The *Bsa*HI-digested fragments were separated on 10% polyacrylamide gel. A complete digestion (denoted mutation) gave 158-bp and 23-bp DNA fragments.

RESULTS

Patients and clinical data

The study included 165 tumor samples from 139 male and 26 female patients with ESCC diagnosed during 1999-2005. The mean age of patients was 63.4 years with a range of 37-91 years (Table 1). Most patients were habitual current smokers (86.7%) and drinkers (72.73%), with most of them (71.5%) reporting both habits. Habitual betel chewing was reported in 17 out of 26 females (65.4%) and in 45 out of 136 males (33.1%).

Table 1 Summary of patient characteristics

Variable	Category	No. of subjects	Frequency (%)
Sex	Male	139	84.2
	Female	26	15.8
Age (yr)	Mean, range	63.4 (37-91)	
Smoking	Never	19	11.6
	Habitual	143	86.7
	Occasional	3	1.8
Drinking	Never	33	20.0
	Habitual	120	72.7
	Occasional	12	7.3
Betel chewing	Never	78	48.1
	Habitual	62	38.3
	Occasional	22	13.6

Table 2 Location and type of mutations

Location	*n* (%)
Exon 5	11 (25.58)
Exon 6	1 (2.33)
Exon 7	9 (20.93)
Exon 8	11 (25.58)
Intron 5	1 (2.33)
Intron 6	8 (18.60)
Intron 8	1 (2.33)
Exon-intron 6	1 (2.33)
Type of mutations	
Transitions	
G:C -> A:T	8 (18.60)
G:C -> A:T at CpG	4 (9.30)
A:T -> G:C	2 (4.65)
Transversions	
G:C -> C:G	13 (30.23)
G:C -> T:A	7 (16.28)
A:T -> T:A	4 (9.30)
Tandem	
GT -> TA	1 (2.33)
Deletion	4 (9.30)

Mutation frequency and patterns

A total of 43 mutations were found in 42 tumors of the 165 samples (25.45%). The representative SSCP gels and sequencing chromatograms are shown in Figure 1.

Twenty-five mutations were missense mutations; one was nonsense, four were frameshift deletions, three were stop codons, and 10 were single base substitutions in the intron region. Mutations in coding sequences were most commonly found in exon 5 (25.58%) and exon 8 (25.58%) (Table 2). Among the 10 intronic mutations found, eight were intron 6 mutations.

Of the five major mutation hot spots of the *p53* gene (codon 175, 245, 248, 273 and 282), mutations at codon 248 were observed in four cases (accounting for 9.3% of all mutations), whereas mutations at other codons were not found.

The types of mutations are shown in Table 2. The most common type was G:C→C:G (30.23%), followed by G:C→A:T (27.90%) and G:C→T:A (16.28%). Surprisingly, eight out of 10 intronic mutations were found at the same location, that is, a G→C substitution at the

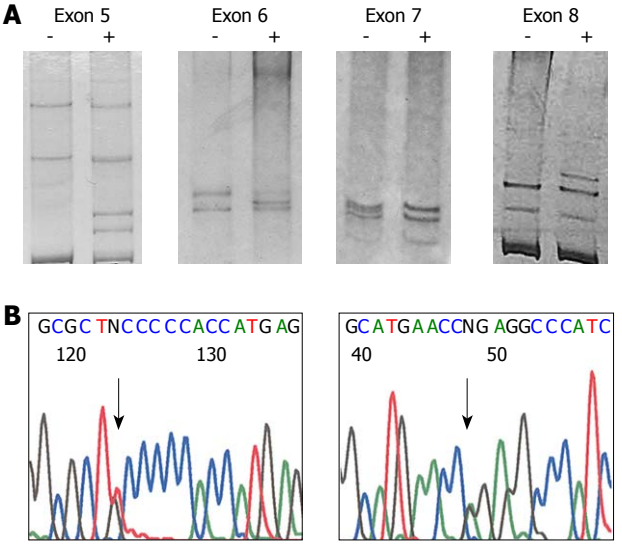

Figure 1 Single-strand conformation polymorphism analysis and direct sequencing of *p53*. A: Single-strand conformation polymorphism of *p53*, exons 5-8 in representative positive cases (+), which showed anomalous bands compared to normal bands in negative cases (-); B: Sequencing chromatograms that represent case E355 (left), which shows TGC-> TTC mutation at codon 176 (arrow) and case E106 (right), which shows CGG-> CAG at codon 248 (arrow).

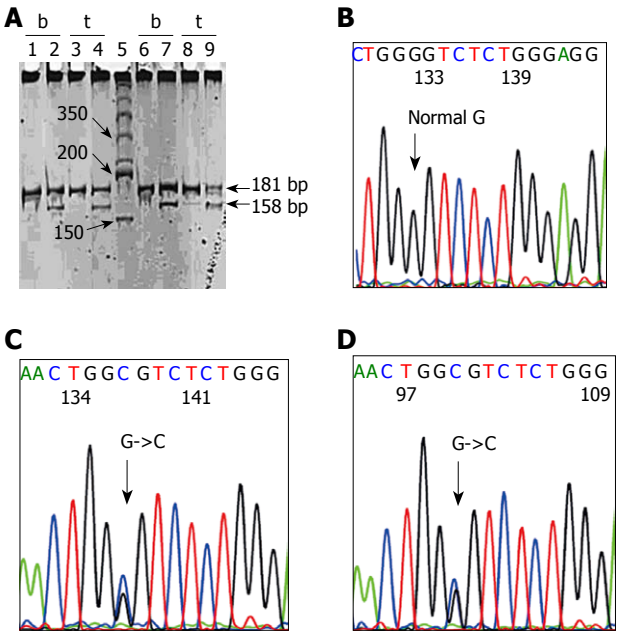

Figure 2 Restriction fragment length polymorphism analysis and direct sequencing to detect intron 6 mutation. A: Detection of the G to C substitution at base 18 after the end of exon 6 by polymerase chain reaction-restriction fragment length polymorphism using *Bsa*HI restriction enzymes. Lane 5 is the size marker. Lanes 1, 3, 6, 8 are uncut 181 bp products of blood (b) and tumor (t) samples of E189 and E199, respectively. Lanes 2, 4, 7, 9 are cut products of the corresponding samples, which show 181 bp and 158 bp fragments, which indicate the presence of a mutation. The 23-bp fragment was not detected in this gel; B-D: Sequencing chromatograms of the corresponding site. Normal sample (B); tumor (C) and blood (D) samples show G to C substitution.

18th base after the last codon of exon 6 (nucleotide 12759, GenBank NC_000017). The details of the mutations of all the cases are shown in Table 3.

Intronic G→C substitution at nucleotide 12759

As a result of the high frequency of intron 6 G→C substitution at 12759 (eight cases), we confirmed these mutations by the RFLP method and the results were similar. We further investigated whether these were germline mutations by examining their matched blood samples through RFLP and direct sequencing. Seven blood samples were available for RFLP and the results denoted a mutation in all of the cases, which suggested germline mutations (Figure 2). The DNA of only four blood samples was available for further direct sequencing and the mutations were confirmed in three out of four samples examined (Figure 2).

We also evaluated functional changes in the p53 proteins of these cases using an immunohistochemistry method (p53 antibody DO-7 clone; DakoCytomation, Carpinteria, CA, USA). Only five cases had adequate tissue for evaluation and the results showed diffuse strong expression in four cases and negative expression in one.

Mutations in relation to clinical factors and exposure

The frequency of mutations in relation to clinicopathological variables is shown in Table 4. Patients younger than 60 years had a significantly higher frequency of *p53* mutations than older patients (38.7% *vs* 17.5%, $P = 0.002$). The mutation frequency was equal in both sexes. In relation to lifestyle habits, the frequency of mutations was slightly higher in non-smokers (36.4%) than smokers (23.8%), and in non-drinkers (33.3%) than drinkers (22.5%). The mutation frequency among betel and non-betel chewers was equal (24.2% and 25.0%). However, there were no statistically significant differences in mutation frequency between exposed and non-exposed patients to all of the three habits.

DISCUSSION

The present study is the third on *p53* mutations in Thai ESCC patients. All of the samples in these three studies were from the same hospital, a university hospital in Songkhla province, Southern Thailand. These studies were conducted at different times, and the present study confirmed the findings of the previous studies and found an additional unique mutation profile.

The present study demonstrated a 25.45% (42/165) frequency of *p53* mutations. This frequency is relatively low compared to those of previous studies; however, wide variations in *p53* mutation frequencies, ranging from 17% to 80%, have been reported. These variations might be related to several factors including the sensitivity of technique used in the detection of the mutations, the length of the examined regions, and the number of cases. However, the most notable factor responsible for the frequency variation could be the difference in mutagens in different populations. A high frequency of *p53* mutation (> 50%) is usually reported in countries with a high incidence of ESCC, such as China and France[14,15], whereas lower frequency of mutation is found in low- or moderate-incidence countries[16-18]. Thailand is a moderate-risk

Table 3 ***p53*** **mutations in esophageal squamous cell carcinoma patients from Songkhla, Southern Thailand**

Case ID	Age (yr)/sex	Exon	Codon	Nucleotide change	AA change	Exposure		
						Smoke	Alcohol	Betel
E111	60/M	5	130-131	3 bp deletion	Frameshift	Yes	Yes	Yes
E303	73/M	5	134-137	10 bp deletion	Frameshift	-	-	-
E006	63/M	5	156	CGC -> CCC	Arg -> Pro	Yes	Yes	No
E271	57/M	5	158	CGC -> CTC	Arg -> Leu	Yes	Yes	No
E072	58/F	5	159	GCC -> CCC	Ala -> Pro	Yes	Yes	Yes
E200	60/F	5	161	GCC -> ACC	Ala -> Thr	Yes	Yes	Yes
E397	56/M	5	167	CAG -> CGG	Gln -> Arg	Yes	Yes	No
E379	66/M	5	168	CAC -> CTC	His -> Leu	Yes	Yes	No
E355	61/M	5	176	TGC -> TTC	Cys -> Phe	Yes	No	Yes
E014	77/M	5	176	TGC -> TAC	Cys -> Tyr	No	Yes	Yes
E022	46/M	5	184	GAT -> AAT	Asp -> Asn	Yes	Yes	Yes
E464	68/M	6	190	CCT -> CTT	Pro -> Leu	Yes	Yes	No
E259	60/M	7	228-232	21 bp deletion	Frameshift	Yes	Yes	No
E320	72/M	7	234	TAC -> TGC	Tyr -> Cys	No	Yes	Yes
E264	52/M	7	238	TGT -> TAT	Cys -> Tyr	Yes	Yes	No
E446	58/M	7	245	GGC -> CGC	Gly -> Arg	Yes	Yes	No
E106	56/M	7	248	CGG -> CAG (CpG site)	Arg -> Gln	Yes	Yes	No
E298	79/F	7	248	CGG -> TGG (CpG site)	Arg -> Trp	Yes	No	Yes
E294	52/M	7	248	CGG -> TGG (CpG site)	Arg -> Trp	Yes	Yes	No
E419	54/M	7	248	CGG -> TGG (CpG site)	Arg -> Trp	Yes	Yes	No
E022	53/M	7	249	AGG -> ATG	Arg -> Met	Yes	Yes	No
E012	74/M	8	266	GGA -> TGA	Gly -> Ter (end)	Yes	Yes	No
E231	54/M	8	272	GTG -> TAG	Val -> Ter (end)	Yes	Yes	Yes
E455	46/M	8	272	GTG -> ATG	Val -> Met	No	Yes	Yes
E449	47/M	8	278	CCT -> TCT	Pro -> Ser	No	Yes	Yes
E002	53/M	8	279	GGG -> GAG	Gly -> Glu	Yes	Yes	-
E027	54/M	8	280	AGA -> AGT	Arg -> Ser	Yes	Yes	No
E444	79/M	8	283	CGC -> CCC	Arg -> Pro	No	Yes	No
E387	74/F	8	286	GAA -> CAA	Glu -> Gln	Yes	Yes	No
E146	62/M	8	287	GAG -> TAG	Glu -> Ter (end)	Yes	Yes	No
E181	58/F	8	287	GAG -> TAG	Glu -> Ter (end)	No	No	No
E408	58/F	8	296	CAC -> CTC	His -> Leu	No	No	No
E462	74/M	Exon-intron 6		21 bp deletion	Affect splice site	Yes	Yes	No
E023	51/F	Intron 5		TGAGC -> TCTGC	-	No	No	Yes
E158	61/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	Occ
E169	53/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	No
E189	60/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	Yes
E199	48/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	Yes
E240	61/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	-
E302	41/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	Occ
E329	63/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	Yes
E435	45/M	Intron 6		GGGG -> GGCG	-	Yes	Yes	No
E409	56/M	Intron 8		ACGAG -> ACTAG	-	Yes	Yes	Yes

Occ: Occasional.

area for ESCC; therefore, the frequency can be expected to be relatively low.

Nevertheless, the two previous studies from Thailand have demonstrated higher frequency of *p53* mutations compared to the present study[12,13]. The first study by Suwiwat *et al*[12] has reported 10 mutations in eight out of 16 (50%) cases. The second study by Tanière *et al*[13] has reported 25 mutations in 23 out of 56 cases (41%). The lower frequency might represent underestimated data, whereas the higher frequency might represent overestimated data. The low frequency of mutations in the present study might have been due to various factors, among which was the fact that we used tumor samples that could have contained both tumor and non-tumor cells, in contrast to the microdissected tumor cells used in the study of Tanière *et al*[13]. With regard to the screening method used, both SSCP and denaturing gradient gel electrophoresis (Tanière study) have been reported to have comparable sensitivity[19]. However, the small number of cases examined could result in over-figured data due to sampling bias.

The present study demonstrated heterogeneous mutation types, which predominantly involved the G:C base pair. This is similar to previous Thai reports except for a relatively higher proportion of G:G to C:G transversion (30% *vs* 23%) and a lower proportion of G:C to A:T transition at CpG (9.3% *vs* 17.14%). The patterns of predominant G:A to A:T transition and G:C to T:A transversion have also been reported in high-risk areas such as China[14,20] and moderate-risk countries such as Japan and India[18,21]. This is different, however, from the high-risk area of Western Europe where a relatively

Table 4 Mutation frequency in relation to clinical factors and exposure *n* (%)

Variables	Mutant *p53*	Wild-type *p53*	*P* value
Sex			
Male	36 (25.9)	103 (74.1)	
Female	6 (23.1)	20 (76.9)	0.762
Age (yr)			
≤ 60	24 (38.7)	38 (61.3)	
> 60	18 (17.5)	85 (82.5)	0.002
Family history			
Yes	6 (37.5)	10 (62.5)	
No	33 (23.7)	106 (76.3)	0.230
Smoking			
Yes	34 (23.8)	109 (76.2)	
No/occasional	8 (36.4)	14 (63.6)	0.207
Drinking			
Yes	27 (22.5)	93 (77.5)	
No/occasional	15 (33.3)	30 (66.7)	0.155
Betel chewing			
Yes	15 (24.2)	47 (75.8)	
No/occasional	25 (25.0)	75 (75.0)	0.908

higher proportion of mutations at the A:T base pair has been reported[22].

The G:C to A:T transition accounted for 28% of all mutations in the present study. One-third of these (4/12 mutations) were G:C to A:T transition at the CpG site, and all were found at the hot spot codon 248. A G:C to A:T transition at the CpG site was thought to have resulted from spontaneous deamination of 5-methylcytosine to form thymine[23], which preferentially occurred at codons 175, 245, 248, 273 and 282 in the *p53* gene. The previous Thai studies have reported transition at the CpG site of codon 175 (one case), 273 (one case) and 248 (three cases)[12,13]. These findings suggest that codon 248 is the most common hot spot codon in Thai ESCC cases.

In reference to the G:C to A:T transition at a non-CpG site, laboratory studies have found that it is the most common mutation caused by alkylating agents, consistent with O^6-methylguanine mispairing with thymine[24]. Mutagenic alkylating N-nitrosamines in tobacco smoke might be responsible for this mutation. In China and India, dietary *N*-nitrosamines might also contribute to this mutation type[20,25]. Our previous study has demonstrated that betel chewing also is a strong risk factor for ESCC in Thailand[9]. Nitroso derivatives from areca alkaloids have been proven to be oncogenic in animal models[26]. They have been found probably to account for the predominant G:A to A:T transition in betel-chewing-related oral cancers[27]. Most of the patients in the current study had a history of drinking and smoking, as well as betel chewing, therefore, smoking and betel chewing might both contribute to the G:C to A:T transition in Thai ESCC patients. However, it is difficult to identify a specific type of mutation with a specific risk factor because the mutation patterns are considerably heterogeneous and most patients have multiple risk habits.

In the present study, we unexpectedly found a high frequency of G to C substitution at the 18th base after the end of exon 6 (nucleotide 12759, GenBank NC_000017). We additionally found that these were germline mutations because similar mutations were also found in their blood samples. We validated these results by a second method, the RFLP.

From the total of 26597 somatic mutation records in the IARC *TP53* database, version R14[28], intronic mutations have been found in 699 records, which represents 2.63% of the total mutations. G to C substitution at nucleotide 12759, similar to the present study, has been found in three cases; two were gastric lymphomas from Hong-Kong[29] and one was small-cell lung carcinoma from Russia[30]. There was a case of ESCC reported to have intron 6 G to C at nucleotide 12758. Surprisingly, this was a case from the study of Tanière *et al*[13], which was the previous study from our hospital. Looking at the details of the mutation in this published article, we found it to be GGGG→GGCG (case 9, Table II)[13], which represents a change at the 18th base after the end of exon 6 or nucleotide 12759, based on the GenBank NC_000017 reference sequence, rather than at nucleotide 12758. Surprisingly, in this case, a similar mutation was also found in the adjacent uninvolved tissue and gastric mucosa, which suggests a germline mutation. These findings suggest that intronic G to C substitution at nucleotide 12759, which might be a germline mutation, is prevalent in Thai ESCC. It should be noted that the cases included in the Tanière study would not have been included in the present study because the periods of sample collection did not overlap (1990-1998 *vs* 1999-2005).

The role of intronic base changes on the function of genes has been questioned. However, some studies have demonstrated alterations in introns or splice donors that affect the expression or function of the *p53* gene[31,32]. In particular, Lehman *et al*[32] have demonstrated functional change of the immortalized lymphoblastoid cell lines derived from familial breast cancer patients who had germline G to C substitution in intron 6 at nucleotide 13964 (or nucleotide 13274 based on the GenBank NC_000017). In addition, immunohistochemical analysis of breast tumors from these patients also has revealed high levels of mutant p53 protein, which suggests a functional mutation. Our results were consistent with this study, which confirms that cases with suspected germline G to C substitution at 12759 have a high level of p53 expression. All this evidence indicates that germline intronic G to C substitution at 12759 is prevalent and associated with inherited risk of ESCC in Songkhla, Thailand. In a recent IARC *TP53* database, version R14[28], this intronic base change has not been reported as any polymorphic sequence variation (polymorphism) or germline mutation. However, as this mutation was not investigated in healthy controls in the current study, any conclusion on the role of this mutation is still limited. Further studies to detect this mutation in healthy controls as well as in familial members of affected patients should be performed.

It is believed that *p53* mutations result from specific carcinogens[11]. In some cancers, such as those of the lung or urinary bladder, the link between risk factors, in particular smoking, and *p53* mutation frequency and/or

pattern have been consistently demonstrated[30,33]. However, such data on esophageal carcinoma are limited and inconsistent[20,34]. Consistent with some of these reports, the present study did not find any association between *p53* mutation frequency and smoking, alcohol consumption or betel chewing. Various reasons could account for the lack of association. ESCC might be associated with many risk factors. This hypothesis is supported by studies from India that have found a significant correlation of *p53* mutation frequency in ESCC with diets rich in nitrosamines[25,35]. In addition, risk of cancer development might be different among exposed individuals due to genetic polymorphism of carcinogen-metabolizing enzymes, which determine individual capacity to detoxify carcinogens. This could modify the relationship between the exposure and gene mutation. Finally, the sample size in the current study could have been too small to detect any significant association between exposure and *p53* gene mutation.

In conclusion, our results have demonstrated that the Thai population, which is in a moderate-risk area for ESCC, has *p53* mutational spectra that are likely related to specific endogenous and exogenous carcinogens. However, a statistically significant relation between the mutation frequency among exposure groups was not demonstrated. We unexpectedly found a high frequency of G to C mutation at intron 6, which might be germline mutations. Further studies are needed to explore the questions arising from the results observed.

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COMMENTS

Background

Cancer of the esophagus is prevalent in some regions of the world including Thailand. It is a dreadful disease that patients may die shortly after diagnosis. Environmental factors as well as familial predisposition have been shown to be associated with the development of this cancer, possibly *via* an alteration of the *p53* tumor-suppressor gene.

Research frontiers

Mutations in the *p53* gene have been implicated to be critical events in the development of various cancers. Significant association between specific exposures and the *p53* mutations has been evident in some cancers, but the data in esophageal squamous cell carcinoma are limited.

Innovations and breakthroughs

The mutation profiles identified are consistent with most previous reports. The mutation types, G:C to C:G (30.2%), G:C to A:T (27.9%) and G:C to T:A (16.3%) were prevalent and likely to be associated with combination of exposures. Exceptionally, a unusually high frequency (8 from 42 cases) of intron 6 mutation (G to C substitution) at nucleotide 12759 was found and they were proofed to be germline mutations.

Applications

The results indicated that a proportion of esophageal cancer in this region is heritable. Further study is to be conducted to identify this specific germline mutation in healthy population and in familial members of the patients. The information would be valuable for designing diagnosis and preventive intervention in high-risk population.

Terminology

An intron is a region within a gene that is not translated into protein. It is transcribed to pre-mRNA and subsequently removed by a process called splicing. A germline mutation is a heritable variation in the lineage of germ cells. Mutations in these cells are transmitted to offspring while those in somatic cells are not. Germline mutations play a key role in genetic diseases and also in certain types of cancer.

Peer review

The authors found intronic *p53* mutation in esophageal squamous cell carcinoma in Southern Thailand, which was considered as a germline mutation. This is a novel finding and interesting.

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BRIEF ARTICLE

Baculovirus vector-mediated transfer of NIS gene into colon tumor cells for radionuclide therapy

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Author contributions: Yin HY and Zhou X performed the majority of experiments; Wu HF performed parts of the experiments; Li B designed parts of the studies; Zhang YF designed the majority of the studies and wrote the manuscript.

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Abstract

AIM: To investigate the feasibility of radionuclide therapy of colon tumor cells by baculovirus vector-mediated transfer of the sodium/iodide symporter (NIS) gene.

METHODS: A recombinant baculovirus plasmid carrying the NIS gene was constructed, and the viruses (Bac-NIS) were prepared using the Bac-to-Bac system. The infection efficiency in the colon cancer cell line SW1116 of a green fluorescent protein (GFP) expressing baculovirus (Bac-GFP) at different multiplicities of infection (MOI) with various concentrations of sodium butyrate was determined by flow cytometry. An *in vitro* cytotoxicity assay was also conducted after infection of SW1116 cells with Bac-NIS. Iodine uptake of Bac-NIS infected SW1116 cells and inhibition of this uptake by sodium perchlorate was examined, and the effect of Bac-NIS-mediated ^{131}I in killing tumor cells was evaluated by cell colony formation tests.

RESULTS: Infection and transgene expression in SW1116 with Bac-GFP were significantly enhanced by sodium butyrate, as up to 72% of SW1116 cells were infected with the virus at MOI of 400 and sodium butyrate at 0.5 mmol/L. No obvious cytotoxicity was observed under these conditions. Infection of SW1116 with Bac-NIS allowed uptake of ^{131}I in these tumor cells, which could be inhibited by sodium perchlorate. The viability of SW1116 cells infected with Bac-NIS was significantly lower than with Bac-GFP, suggesting that NIS gene-mediated ^{131}I uptake could specifically kill tumor cells.

CONCLUSION: Baculovirus vector-mediated NIS gene therapy is a potential approach for treatment of colon cancer.

Key words: Colon cancer; Baculovirus; Sodium iodide symporter; Radionuclide therapy; Iodine radioisotopes

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INTRODUCTION

Colon cancer is one of the most common malignant gastrointestinal tumors with a high morbidity and mortality. Currently, surgery and chemotherapy are the primary methods for treating colon cancer. However, surgical removal of the tumor is not an option for 30%-40% of these patients upon diagnosis because of metastasis;

meanwhile, chemotherapy shows a relatively high incidence of severe side effects. Therefore, it is important to find alternative ways to improve the prognosis of patients with colon cancer.

The cloning of the sodium/iodide symporter (NIS) gene and subsequent studies of its properties have resulted in a new approach to targeted radiotherapy of malignant cancers. NIS is a transmembrane glycoprotein located in the basal membrane of thyroid follicular cells. It can effectively co-transport two Na^+ and one I^- into cells[1], and this transportation of iodine is an active process against the chemical gradient. The uptake of radioactive ^{131}I can be achieved by the expression of the NIS protein in tumor cells *via* vector-mediated gene transfer, and the radiotherapeutic destruction of the tumor occurs by emission of β rays from ^{131}I.

Recently, several researchers have treated tumors by delivering the NIS gene *via* vectors based on retroviruses[2], adenoviruses[3] and lentiviruses[4], and achieved a certain level of therapeutic effect. For the purpose of gene transfer, an ideal vector would have high transduction efficiency and robust protein expression so that the uptake of radioactive ^{131}I in tumor cells will reach an effective radiotherapeutic dose. The adenovirus vector system does in fact have high transduction efficiency, but its high immunogenicity and cytotoxicity prevent its further application *in vivo*. The major concern regarding the retroviral or lentiviral system is that the transgenes are easily integrated into the genomic DNA of host cells, which may cause changes in the target genome expression and result in oncogenicity *in vivo*. Moreover, long-term gene expression is not necessary in therapeutic gene-mediated radiotherapy.

We previously performed a pilot study using a baculovirus vector to deliver the NIS gene as a reporter[5]. Our results showed that the large NIS gene could be inserted into the recombinant baculovirus. A follicular thyroid cancer cell line, FTC-133, infected with recombinant green fluorescent protein (GFP) baculovirus previously showed a high expression of GFP, which lasted approximately seven days[5]. Considering the above characteristics, we reasoned that a recombinant NIS gene baculovirus may be beneficial for mediating ^{131}I uptake and the killing of tumor cells. Furthermore, since baculoviruses can only infect arthropods, this vector is highly safe for mammals. In fact, recombinant baculoviruses have been widely used in mammalian cells as a gene transfer vector because of these advantages[6]. However, no reports are available on baculovirus vector-mediated radiotherapy for cancer.

In this study, the recombinant sodium/iodide symporter gene baculovirus (Bac-NIS) was generated and used to infect colon cancer cells to investigate the expression and function of NIS in these cells and to provide an experimental basis for the NIS gene-mediated targeted radiotherapy of colorectal cancer.

MATERIALS AND METHODS

Construction of baculovirus vectors

The baculovirus vector pFBGFPR (kindly provided by the Molecular Institute of the University of Hong Kong) was digested with restriction endonucleases Acc I and Age I. The digested ends of the vector were filled in with Klenow fragment, yielding a 6 kb pFB fragment which was then purified. The target gene was prepared by digesting the recombinant plasmid pCDNA-NIS (kindly provided by Professor Sissy Jhiang of Ohio University, USA) with restriction endonucleases *Bam*H I and *Xho* I and filled in with Klenow kinase to obtain the 2.3 kb NIS gene fragment. The purified NIS target gene fragment was ligated to the phosphatase-treated pFB vector using T4 bacteriophage DNA ligase. The resulting plasmid was transformed into DH5α competent bacteria (Tiangen Biotech Co., Ltd., Shanghai) and screened on ampicillin Luria Burtani (LB) culture plates. White colonies were selected and screened for the correct orientation of the inserted target gene fragment by sequencing. The confirmed recombinant baculovirus pFBNIS plasmid DNA was amplified and purified.

Preparation of recombinant NIS and GFP baculoviruses

The baculovirus vector plasmid pFBGFPR and the constructed recombinant plasmid pFBNIS were transformed into DH10 competent bacteria (Invitrogen). The successfully transpositioned white colonies were selected and amplified. Bacmid plasmids expressing the NIS gene were extracted and transfected into Spodoptera frugiperda (sf9) cells using Lipofectamine 2000 (Invitrogen). The supernatant of the transfected cells containing the virus was collected 5-7 d later. Sf9 cells were infected at a multiplicity of infection (MOI) of 0.1 and harvested after 5-7 d in culture by centrifuging at 10000 × *g* for 15 min. The resulting supernatant was collected, centrifuged again at a high speed of 10000 × *g* at 4℃ for 5 min, sterilized with a 0.45 μm filter and preserved at 4℃ in the dark. Plaque assays were used to determine the titer of baculovirus stocks.

SW1116 cell culture

SW1116 (human Caucasian colon adenocarcinoma) was cultured in DMEM (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) and 1% penicillin/streptomycin (Gibco-BRL) in a humidified environment with 5% CO_2 at 37℃.

Baculovirus infection of SW1116 cells

SW1116 cells were seeded in 6-well or 24-well plates with DMEM with 10% FBS at least 24 h before infection. The medium was changed to fresh serum-free DMEM just before infection. After infection with the baculovirus vector at indicated MOIs, the cells were incubated with serum-free DMEM for 4 h, followed by addition of FBS to the final concentration of 10%. All infections were performed in triplicate.

Effects of sodium butyrate on the transduction efficiency and fluorescence intensity of SW1116 cells

SW1116 cells were seeded in 6-well plates at a density of 1 × 10^5 cells/well and cultured with DMEM without serum for 24 h. Recombinant green fluorescent protein

gene baculovirus (Bac-GFP) was then added at an MOI of 400, and sodium butyrate was added at different concentrations (0, 0.5, 1 and 2 mmol/L) simultaneously. All experiments were performed in triplicate. Cells were incubated at 37℃ for 4 h after treatment, and FBS was added to the final concentration of 10% and incubated for another 24 h. The expression of GFP and the mean fluorescence intensity were determined by flow cytometry. The excitation wavelength of blue laser was 488 nm, and the detection wavelength was 520 nm.

Infection efficiency of Bac-GFP in SW1116 cells

SW1116 cells were seeded in 6-well plates at a density of 1×10^5 cells/well, and cultured with serum-free DMEM for 24 h. Bac-GFP was added at the MOI of 0, 100, 200, or 400. The final concentration of sodium butyrate was 0.5 mmol/L, and cells without sodium butyrate were used as negative controls at various MOI of Bac-GFP. All experiments were performed in triplicate. After treatment, the cells were incubated at 37℃ for 4 h, and FBS was added to reach the final concentration of 10% and incubated at 37℃ for another 24 h. The expression of GFP was observed under an inverted phase-contrast fluorescence microscope. The expression of GFP and the mean fluorescence intensity were detected by flow cytometry.

Effects of Bac-GFP infection and sodium butyrate on the cytotoxicity of SW1116 cells

SW1116 cells were seeded in 6-well plates at a density of 1×10^4 cells/well and cultured with serum-free DMEM for 24 h. Bac-GFP was added at the MOI of 0, 50, 100, 200 or 400. For testing the effect of sodium butyrate, the final concentration was 0, 0.5, 1, and 2 mmol/L with Bac-GFP at an MOI of 400, and wells without sodium butyrate were used as the control well at an MOI of 400. After treatment, the cells were incubated at 37℃ for 4 h, FBS was then added to reach the final concentration of 10% and incubated at 37℃ for another 24 h. Twenty microliters of 5 g/L MTT (Beyotime Co., Ltd., Shanghai) were added to each well and incubated for another 4 h. Subsequently, 100 μL DMSO was added, and the plate was gently shaken to completely dissolve the blue-violet precipitate. The absorption at 490 nm (A_{490}) was determined using a microplate reader. Cell viability = test well A_{490}/control well $A_{490} \times 100\%$.

Determination of iodine uptake of SW1116 cells infected with Bac-NIS

SW1116 cells were seeded in 6-well plates at a density of 5×10^4 cells/well and cultured with serum-free DMEM for 24 h. Bac-NIS was added at the MOI of 0, 50, 100, 200 or 400. The final concentration of sodium butyrate was 0.5 mmol/L, and cells without sodium butyrate were used as negative controls at various MOI of Bac-NIS. All experiments were performed in triplicate. After treatment, cells were incubated at 37℃ for 4 h, FBS was then added to reach the final concentration of 10% and incubated at 37℃ for another 24 h. The cells were washed twice with bHBSS solution (HBSS solution buffered with HEPES solution to achieve a pH 7.3), and then 0.5 mL bHBSS solution containing 0.1 μCi Na ^{125}I (Shanghai Hinko Co.) with 1 μmol/L NaI were added into each well as reported by Weiss *et al*[7]. Cells were incubated at 37℃ for 30 min, washed with cold bHBSS solution three times and incubated for another 20 min with 1 mL 100% cold ethanol. The samples were then collected to determine the radioactive counts per minute (cpm) by a well γ-counter.

Dynamic determination of ^{125}I uptake of SW1116 cells

SW1116 cells were seeded in 6-well plates at a density of 5×10^4 cells/well and cultured with serum-free DMEM for 24 h. Bac-NIS and Bac-GFP were added at the MOI of 400 respectively. The final concentration of sodium butyrate was 0.5 mmol/L, and Bac-GFP was used as negative controls. All experiments were performed in triplicate. After treatment, cells were incubated at 37℃ for 4 h, FBS was then added to reach the final concentration of 10% and incubated at 37℃ for another 24 h. Cells were washed twice with bHBSS solution, and 0.5 mL bHBSS solution containing 0.1 μCi Na ^{125}I with 1 μmol/L NaI were added into each well, incubated for 5, 10, 15, 30, 60, 90, or 120 min, washed with cold bHBSS solution three times, and incubated for another 20 min with 1 mL 100% cold ethanol. The samples were then collected to determine the radioactive cpm by a well γ-counter.

Inhibition of iodine uptake by $NaClO_4$

SW1116 cells were seeded in 6-well plates at a density of 5×10^4 cells/well and cultured with serum-free DMEM for 24 h. Bac-NIS was added at the MOI of 400. The final concentration of sodium butyrate was 0.5 mmol/L. All experiments were performed in triplicate. After treatment, cells were incubated at 37℃ for 4 h, and FBS was added to reach the final concentration of 10% and incubated at 37℃ for another 24 h. Cells were washed twice with bHBSS solution. Subsequently, 0.5 mL bHBSS solution containing 0.1 μCi Na ^{125}I was added into each well with $NaClO_4$ at concentrations of 0, 30, or 300 μmol/L. After incubation for 30 min, the cells were washed with cold bHBSS solution three times and incubated for another 20 min with 1 mL 100% cold ethanol. The samples were then collected to determine the radioactive cpm by a well γ-counter.

^{131}I-mediated killing of tumor cells in cell colony formation test

SW1116 cells were inoculated in 6-well plate at a density of 5×10^4 cells/well and cultured with serum-free DMEM for 24 h. Bac-NIS and Bac-GFP were added at an MOI of 400 with sodium butyrate at 0.5 mmol/L respectively. Bac-GFP was used as the negative control. The same volume of bHBSS solution was added into a blank well as a control. All experiments were performed in triplicate. After treatment, cells were incubated at 37℃ for 4 h, and FBS was added to reach the final concentration of 10% and incubated at 37℃ for another 24 h. Cells were washed twice with bHBSS solution. After 0.5 mL bHBSS solution containing 0.1 μCi Na ^{125}I was added into each well and incubated for another 6 h, the cells were washed

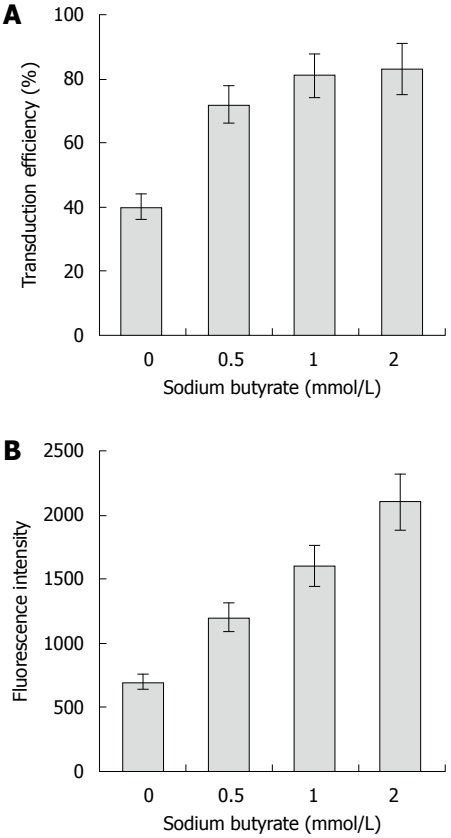

Figure 1 Effects of sodium butyrate on infection efficiency of SW1116 cells. The transduction efficiency (A) and fluorescence intensity (B) of recombinant green fluorescent protein gene baculovirus (Bac-GFP) infection of SW1116 cells at multiplicities of infection (MOI) of 400 in the presence of sodium butyrate at the indicated concentrations. Error bars represent standard deviations.

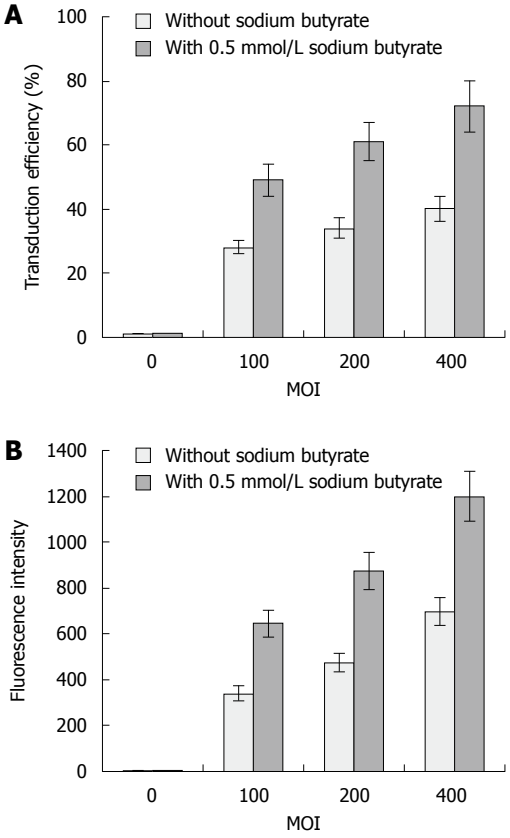

Figure 2 Recombinant green fluorescent protein gene baculovirus infection efficiency in SW1116 cells with or without sodium butyrate. The transduction efficiency (A) and fluorescence intensity (B) of recombinant green fluorescent protein gene baculovirus (Bac-GFP) infection of SW1116 cells at different multiplicities of infections (MOIs) as indicated with or without 0.5 mmol/L sodium butyrate. The infection efficiency (%) and the fluorescence intensity of GFP were determined by flow cytometry. Error bars represent standard deviations.

twice with bHBSS solution, digested, counted, seeded in a 6-well plate (1000 cells/well) and incubated at 37℃ for additional 7 d. Cells were then washed three times with bHBSS solution and stained with crystal violet solution (Sigma). Cell viability was determined in the infected cells by counting colonies visible to the naked eyes and calculated as a percentage of the colonies that were treated with only bHBSS[8].

Statistical analysis

All data are presented as mean ± SD. Differences were considered significant at $P < 0.05$. Statistical analysis was performed using SPSS 11.0 software.

RESULTS

Sodium butyrate enhances infection efficiency of Bac-GFP and expression of the reporter protein

The transduction efficiencies of baculovirus in SW1116 cells were 39%, 71%, 80% and 83% at an MOI of 400 with concentrations of sodium butyrate of 0, 0.5, 1 or 2 mmol/L, respectively. This result indicated that sodium butyrate could significantly improve the transduction efficiency of baculovirus in SW1116 cells, achieving high levels of transduction at concentrations above 0.5 mmol/L. The fluorescence intensity of the infected SW1116 cells increased along with sodium butyrate in a dose-dependent manner, suggesting that the expression of the reporter protein was enhanced by the sodium butyrate (Figure 1).

Transduction efficiency of Bac-GFP at various MOI in SW1116 cells

The transduction efficiency (Figure 2A) of baculovirus (Bac-GFP) in the SW1116 tumor cells gradually increased, and the fluorescence intensity (Figure 2B) was also enhanced with increasing MOI. Additionally, sodium butyrate could significantly improve the transduction efficiency and fluorescence intensity of tumor cells by Bac-GFP at different MOI (Figure 2). The sodium butyrate-mediated transduction efficiency of baculovirus inoculated at the MOI of 400 in SW1116 cells was 72%.

Cytotoxicity of SW1116 cells infected with baculovirus at different MOIs with various concentrations of sodium butyrate

There was no obvious change in cell viability with the dose escalation of recombinant baculovirus (Figure 3A). There was no significant cell death observed at the highest MOI of 400. These results suggested that the baculovirus itself had no obvious cytotoxic effects on the tumor cells.

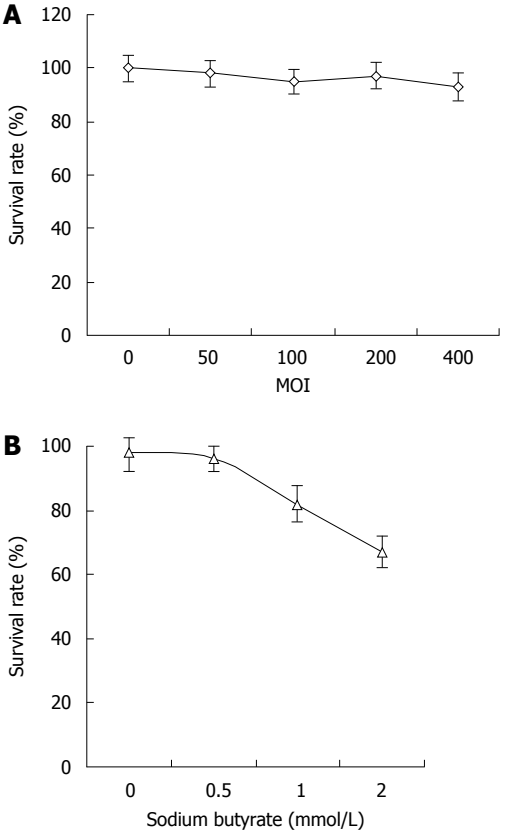

Figure 3 Cytotoxic effects of baculovirus infection and sodium butyrate in SW1116 cells. A: The cytotoxic effects in SW1116 infected with recombinant green fluorescent protein gene baculovirus (Bac-GFP) at different multiplicities of infection (MOI); B: The cytotoxic effects of sodium butyrate at various concentrations in SW1116 cells infected with Bac-GFP at MOI of 400.

On the other hand, the tumor cell viability decreased with increasing concentrations of sodium butyrate with virus at the MOI of 400 (Figure 3B). However, the cell viability did not change significantly when the concentration of sodium butyrate was lower than 0.5 mmol/L.

Determination of iodine uptake in infected SW1116 cells

The radioiodine uptake of tumor cells increased with increasing doses of Bac-NIS, and this uptake was significantly enhanced by sodium butyrate (Figure 4).

Dynamic determination of ^{125}I uptake of SW1116 cells

The radioiodine uptake of infected tumor cells increased over the incubation time with radioiodine which reached the peak at 30 min with Bac-NIS at the MOI of 400 and sodium butyrate at 0.5 mmol/L (Figure 5). This result suggested that the NIS protein expressed in transfected tumor cells could perform its normal function in iodine uptake.

Inhibition of iodine uptake by $NaClO_4$

NIS-mediated iodine uptake in thyroid tissues can be specifically inhibited by ClO_4^-. In the present study, we tested the ability of ClO_4^- at various concentrations to inhibit the iodine uptake by tumor cells expressing the NIS protein. Our results showed that the radioiodine uptake

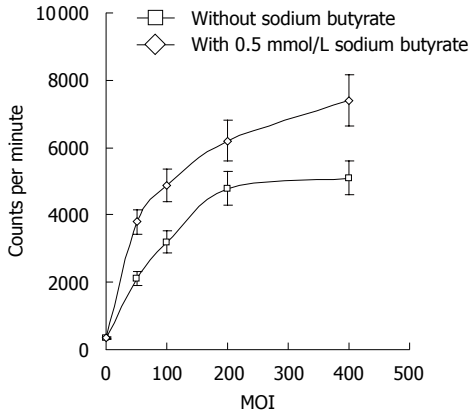

Figure 4 Iodine uptake of SW1116 cells after infection by recombinant sodium/iodide symporter gene baculovirus in the absence or presence of sodium butyrate. ^{125}I iodine uptake (counts per minute) was determined in SW1116 cells infected with recombinant sodium/iodide symporter gene baculovirus at indicated multiplicities of infection (MOI) with or without 0.5 mmol/L sodium butyrate 30 min after exposure to ^{125}I. Error bars represents standard deviations.

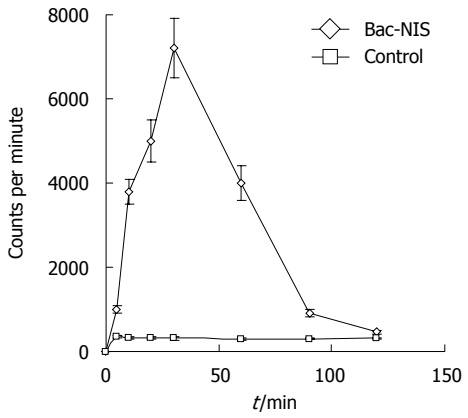

Figure 5 Dynamic changes of iodine uptake in recombinant sodium/iodide symporter gene baculovirus infected SW1116 cells. SW1116 cells were infected with recombinant sodium/iodide symporter gene baculovirus (Bac-NIS) at 400 multiplicities of infection, and iodine uptake (counts per minute) was determined over time when incubated with Na^{125}I and NaI. Error bars represent standard deviations.

of SW1116 cells was significantly reduced with escalating doses of ClO_4^- (Figure 6), indicating that the function of the expressed NIS protein in the infected tumor cells could also be inhibited by the presence of $NaClO_4$.

^{131}I-mediated killing of tumor cells

In order to evaluate the killing effect of ^{131}I in infected tumor cells, a cell colony formation test was performed with cells infected by Bac-GFP as the control. The results showed that the cell viability of SW1116 cells infected with Bac-NIS was reduced to about 20%, and that of SW1116 cells infected with Bac-GFP (70%) was similar to that of the non-infected group (76%) (Figure 7). These observations suggested that NIS-mediated tumor cells could be effectively and specifically killed by ^{131}I.

DISCUSSION

Since 1996 when Dai *et al*[9] and Smanik *et al*[10] cloned the

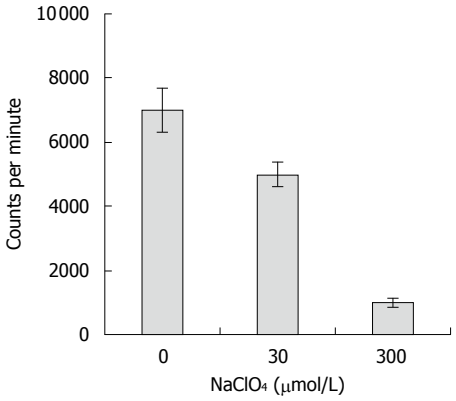

Figure 6 Inhibition rate of $NaClO_4$ in radioiodine uptake in tumor cells after infection. Radioiodine uptake was inhibited by pre-treatment of the SW1116 tumor cells with sodium perchlorate at various concentrations for 30 min. Error bars represent standard deviations.

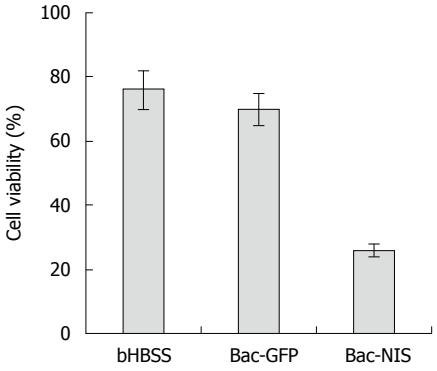

Figure 7 Survival rates (%) of SW1116 cells treated with $Na^{131}I$. Cell viability of infected SW1116 cells was determined after treatment with $Na^{131}I$, SW1116 cells were treated with bHBSS, recombinant green fluorescent protein gene baculovirus (Bac-GFP) with 0.5 mmol/L sodium butyrate, and recombinant sodium/iodide symporter gene baculovirus (Bac-NIS) with 0.5 mmol/L sodium butyrate.

rat and human NIS genes, respectively, researches on the function of these genes have progressed with a breakthrough in the understanding of their role in iodine uptake of thyroid cells. NIS can mediate the active transport of iodine ions. In addition to thyroid tissues, NIS is expressed at low levels in salivary glands, gastric mucosa tissues, breast tissues in secretory phase and other tissues[11]. The ^{131}I therapy for differentiated thyroid carcinoma is performed by expressing the NIS gene in clinical applications.

There have been significant advances in the field of radiotherapy by NIS gene transfer using either non-viral or viral vectors in recent years. Boland *et al*[12] investigated the use of targeted radiotherapy in several tumor cell lines (including cervix cancer, breast cancer and prostate carcinoma) by adenovirus-mediated NIS gene transfer and showed uptake of iodine in tumor cells. Sieger *et al*[13] screened stable cell lines expressing the NIS gene from a retrovirus-infected hepatoma cell line (MH3924A) and showed significant radioiodine uptake as well. While Haberkon *et al*[14] found a low absorbance of radioactive iodine in infected rat prostate carcinoma cells by a retrovirus expressing the NIS gene due to the rapid clearance of iodine in transplanted tumors, Dingli *et al*[15] achieved considerable therapeutic effects by lentivirus-mediated NIS gene transfer in myeloma cells. All of these studies suggest that NIS gene is a promising candidate for targeted tumor radiotherapy.

Presently, viral vectors most commonly used for gene transfer are based on adenoviruses, retroviruses and lentiviruses. Adenoviruses can induce strong host immune responses[16], and antibodies against adenovirus has been found in 97% of the population[17]. These pre-existing antibodies against the viral vector can inhibit the efficiency of gene transfer. Furthermore, transgenes carried by lentiviruses and retroviruses can randomly insert into the genomic DNA of the host cells and potentially activate oncogenes in normal tissues.

Therefore, it is critical to choose a proper vector system to deliver the gene of interest in gene therapy. The baculovirus vector system has a high transduction efficiency and protein expression in mammalian cells[18]. It is a replication competent vector that displays less cytotoxicity to mammalian cells when compared with other viral vectors. Because the baculovirus DNA does not integrate into the host genome, baculovirus vectors have an extremely high biological safety profile. Since baculoviruses also have very species-specific tropisms among the invertebrates, there would be virtually no pre-existing neutralizing antibody and specific T cells in mammals to prevent the application of these vectors *in vivo*. Meanwhile, the packaging capacity of baculovirus is about 38 kb, allowing insertion and expression of large genes, and high titer virus stocks can easily be produced[19]. All these advantages show that the baculovirus is a promising vector for use in gene transfer applications.

In this study, we successfully cloned and purified baculovirus with the NIS gene driven by the cytomegalovirus immediate-early gene promoter. Some studies have shown that the transduction efficiency of recombinant baculovirus can be increased by adding sodium butyrate in SW1116 cells. Infection of Bac-NIS in SW1116 cells showed that it can uptake iodine, and that this process can be specifically inhibited by sodium perchlorate. This suggested that the NIS protein expressed by baculoviruses functioned similarly to the natural NIS protein. The proliferation of SW1116 cell was significantly lower in cells infected with Bac-NIS than those infected with Bac-GFP, indicating that tumor cells infected with Bac-NIS could specifically uptake ^{131}I, and as a result the emitted β-rays could kill the target cells effectively. These experiments provided solid evidence for the feasibility of radionuclide therapy for colon cancer.

The expression level of an exogenous gene positively correlates with the amount of stable transgenes in host cells, and the regulation of the transgene in mammalian cells is the key to its expression. Modifications of the transgenes such as acetylation, methylation and/or the chromatin compactness may play important roles in the expression of the transgenes carried by the baculovirus. Sodium butyrate is a four-carbon short-chain fatty acid derived from dietary fiber in the intestinal tract *via* bacte-

rial fermentation. The concentration of sodium butyrate is very high in the intestinal tract of healthy individuals especially those with a high-fiber diet. Sodium butyrate is a histone deacetylase inhibitor that has been shown to relax chromatin by inducing the acetylation of chromosomes which can in turn facilitate gene expression. Furthermore, it can also significantly improve the transduction efficiency of baculovirus and the expression level of exogenous genes[19]. In this study, we found that sodium butyrate could significantly improve the transduction efficiency of baculovirus and the expression of the NIS transgene in SW1116 cells which led to a significant killing effect (viability = 20%) of SW1116 tumor cells. Therefore, baculovirus-mediated gene transfer of the NIS gene allows targeted radiotherapy for intestinal tumors.

The therapeutic effect of radioiodine therapy depends on the absorbance of target tissues which are affected by the effective half-life of ^{131}I in tumors, the total mass of tumor and the total absorbed dose of ^{131}I. However, iodine uptake in SW1116 cells mediated by NIS gene transfer is rapidly cleared from the body. This is different from the observation in thyroid cells due to the absence of thyroid peroxidase (TPO) in SW1116 cells. Therefore, the organification of inorganic iodine taken up by SW1116 cells to produce thyroid hormone for storage cannot take place, ultimately affecting the therapeutic effect for tumors that can be achieved with ^{131}I. Thus, an important issue in NIS gene-mediated tumor therapy is the improvement of the retention of ^{131}I in tumor cells. Huang *et al*[20] transfected NIS and the TPO gene together into non-small cell lung cancer cell lines and found that the rate of clearance of iodine was reduced. However, it is still controversial whether or not the organification of iodine mediated by TPO would increase the retention of iodine[21].

Various approaches to improve radiotherapy have been reported. It has been shown that NIS-mediated ^{188}Re (Rhenium) radiating β-rays [22] and ^{211}At (Astatine) radiating α-rays[23] in various tissues resulted in better radiotherapeutic effects than with ^{131}I due to higher radiation energy deposition. Additionally, as the serum complement system is the primary inhibitory factor of baculovirus-mediated gene transfer, some researchers have created certain modifications in baculovirus to prevent inactivation by complement in serum. For example, human decay-accelerating factor expressed on the envelope of the virion could render recombinant baculovirus resistant to the complement system, and the expression of transgene could be detected when the modified baculovirus was injected into murine hepatic parenchyma[24]. The inhibition of serum inactivation can also be achieved by injecting complement inhibitors such as cobra-venom factor prior to baculovirus vector delivery[25]. Thus, baculovirus vectors will provide a new approach for the gene therapy of malignant tumors especially when the related techniques are improved.

COMMENTS

Background

Sodium/iodide symporter (NIS) is a transmembrane glycoprotein located in the basal membrane of thyroid follicular cells. It can effectively co-transport two Na^+ and one I^- into cells, and this transportation of iodine is an active process against the chemical gradient. The uptake of radioactive ^{131}I can be achieved by the expression of the NIS protein in tumor cells *via* vector-mediated gene transfer, and the radiotherapeutic destruction of the tumor occurs by emission of β rays from ^{131}I.

Research frontiers

It is a new approach to use NIS gene in radiotherapeutic destruction of the tumor. The authors adopted Bac-NIS to transduce colon cancer cell line, SW1116, to investigate the feasibility of NIS as a therapeutic gene in ^{131}I mediated cell killing. There is currently no report on this.

Innovations and breakthroughs

This is the first report about the baculovirus vector-mediated radiotherapy for colon cancer cell line. NIS was used as target gene, and the results showed that the transduction of baculovirus in cancer cells increased in the presence of sodium butyrate and cancer cells infected with Bac-NIS could be rendered ^{131}I uptake and specifically killed by β-rays from ^{131}I.

Applications

Baculovirus vectors will provide a new approach for the gene therapy of malignant tumors especially when the related techniques are improved.

Terminology

NIS is a transmembrane glycoprotein located in the basal membrane of thyroid follicular cells.

Peer review

^{131}I uptake in cancer cell mediated by NIS gene transfer is rapidly cleared. The therapeutic effects of radiotherapy will be improved with the further research in this field.

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BRIEF ARTICLE

Patterns of lymph node metastasis are different in colon and rectal carcinomas

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Abstract

AIM: To describe patterns of lymph node metastasis in invasive colon and rectal carcinomas.

METHODS: Clinical data of 2340 patients with colorectal carcinoma (stage Ⅰ to Ⅲ) who received radical resection, was retrospectively reviewed. Of the 2340 patients, 1314 patients suffered from rectal carcinoma and 1026 from colon carcinoma. Patients with rectal cancer who received neoadjuvant chemoradiation therapy were excluded. Statistical analysis was performed using Mann-Whitney, χ^2 and Cochran's and Mantel-Haenszel tests (SPSS 15.0). A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS: Lymph node retrieval in the rectal carcinoma group was significantly lower than that in the colon carcinoma group ($P < 0.001$), while positive lymph node retrieval in the rectal carcinoma group was significantly higher than that in the colon carcinoma group ($P < 0.001$). The proportion of lymph node positive (N+) cases was higher (patients with one or more positive lymph nodes) in the rectal carcinoma group ($P = 0.004$). The number of N+ cases was compared at different T stages (T1-T4) to eliminate background bias and the results were confirmed ($P < 0.001$). In addition, the lymph node ratio (the ratio of number of positive lymph nodes over the number of lymph nodes examined) of stage Ⅲ cases in the rectal carcinoma group was significantly higher than that in the colon carcinoma group ($P < 0.001$).

CONCLUSION: Rectal carcinomas seem more prone to metastasize to the lymph nodes than colon carcinomas, which may be of potential clinical significance.

Key words: Lymph node; Metastasis; Colon; Rectum; Neoplasms

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INTRODUCTION

Lymph node metastasis in colorectal cancer significantly in-

fluences patient management and prognosis[1-4]. Therefore, the number of positive lymph nodes is regarded as an index to determine different N stages in the extensively accepted colorectal cancer tumor node metastasis (TNM) staging[5,6]. As to the issues of the minimal number of lymph nodes examined[6-9] and lymph node metastasis[6,10,11], colon cancers and rectal cancers are usually treated as a single entity. Some researchers pointed out that the rate of lymph node metastasis in colon cancer and rectal cancer might be different[12,13]. The present study was attempted to describe the possible differences in patterns of lymph node metastasis between colon and rectal tumors so as to provide useful information for more effective clinical treatment and prognostic assessment of colon and rectal carcinomas.

MATERIALS AND METHODS

Patient selection

Patients with invasive colorectal cancer who received surgical treatment at Changhai Hospital between January 2000 and June 2008 were identified from our prospectively collected database as approved by the Institutional Review Board (IRB). Patient characteristics, examination records, operative and pathologic reports were reviewed. The following data were collected from the patients' charts: (1) Demographic data (age and gender); (2) Preoperative examinations including colonoscopy, abdominal ultrasound and chest X-ray; (3) Preoperative adjuvant treatment; (4) Operative records; and (5) Postoperative pathology: tumor grade [low grade (equal to well and moderately differentiated) or high grade (equal to poorly differentiated and undifferentiated)]; tumor size (refers to the maximum dimension of the primary tumor measured in the operative specimen), T stage (depth of invasion), and N stage (the number of total and positive lymph nodes)[5]. Based on the lymph node status, the patients were divided into node positive (N+) and node negative (N-). N+ patients included all patients with one or more positive lymph nodes (N1 and N2), and N- patients included all patients without positive lymph nodes on final pathological analysis.

Inclusion criteria

Inclusion criteria were primary invasive colorectal cancer confirmed by preoperative pathology and treated with radical operation.

Exclusion criteria

Exclusion criteria were recurrent colorectal tumors, Tis tumors, R1 or R2 resection [The resection margin was considered positive (R1 resection) when the invasive tumor was present in the resection margin or the distance between the tumor and the resection margin was less than 1 mm; R2 resection refers to incomplete tumor resection with gross residual tumor that was not resected]. Patients with rectal cancer who received neoadjuvant chemoradiation or with stage Ⅳ colorectal carcinoma, patients who lacked complete information, and patients with synchronous diseases (such as total colectomy for familial adenomatous polyposis) that might affect lymph node harvest were also excluded.

Procedures

For rectal carcinoma, surgery was performed using a standardized surgical procedure with sharp mesorectal dissection techniques as described by Heald *et al*[14]. Standard colectomy and regional lymphadenectomy (right hemicolectomy, transverse colectomy, left hemicolectomy and sigmoid colectomy) were performed according to the location of colon cancer.

Pathology

The surgical specimens were fixed in 10% buffered neutral formalin. The traditional inspection and palpation method was applied for lymph node harvest. No adjuvant technique such as fat clearance was used to help identify lymph nodes.

Statistical analysis

Statistical analysis was performed using SPSS Software system (version 15.0, Chicago, IL). Mann-Whitney test was employed to numerical data that did not show a normal distribution (age, total number of lymph node examined, the number of positive lymph node) or ordinal variables (T stage and N stage). Comparison of proportions was analyzed by χ^2 test. Cochran's and Mantel-Haenszel tests were applied to compare the difference in the number of N+ patients in different tumor groups to stratify the data by T stage. Results were expressed as median (range). A two-tailed P value of < 0.05 was considered statistically significant. The study was reviewed and approved by Changhai Hospital IRB.

RESULTS

Patient population

A total of 2340 patients were identified, including 1314 patients with rectal carcinoma, and 1026 patients with colon carcinoma involving the cecum in 63 cases, ascending colon in 242 cases, hepatic flexure in 118 cases, transverse colon in 67 cases, splentic flexure in 40 cases, descending colon in 79 cases, and sigmoid colon in 417 cases.

There were 977 females (41.8%) and 1363 males (58.2%) with a median age of 61 (51-71) years. The median tumor size was 4.5 (3.5-6.0) cm. The percentages of stage Ⅰ, Ⅱ and Ⅲ tumors were 19.5%, 41.7% and 38.8% respectively.

Comparison of tumor characteristics and the total number of lymph nodes examined between rectal and colon carcinoma groups

The tumors in the rectal carcinoma group were smaller than those in the colon carcinoma group ($P < 0.001$). In addition, there were more low-grade tumors in the rectal carcinoma group ($P = 0.002$). The total lymph node number in the rectal carcinoma group was lower than that in the colon carcinoma group ($P < 0.001$).

Table 1 Comparison between rectal cancer and colon cancer groups *n* (%)

Clinicopathologic factors	Rectal cancer (*n* = 1314)	Colon cancer (*n* = 1026)	*P* value
Gender			
Male	797 (60.7)	566 (55.2)	0.008
Female	517 (39.3)	460 (44.8)	
Age (yr)	59 (50-68)	64 (53-72)	< 0.001
Grade			
Low	1125 (85.6)	830 (80.9)	0.002
High	189 (14.4)	196 (19.1)	
Tumor size (cm)	4.0 (3.0-5.5)	5.0 (4.0-6.0)	< 0.001
Total nodes	9 (7-12)	10 (8-13)	< 0.001
Positive nodes	3 (1-5) (*n* = 544)	2 (1-4) (*n* = 364)	< 0.001
LNR	0.31 (0.14-0.60) (*n* = 544)	0.22 (0.13-0.50) (*n* = 364)	< 0.001
N stage			
N0	770 (58.6)	662 (64.5)	< 0.001
N1	338 (25.7)	267 (26.0)	
N2	206 (15.7)	97 (9.5)	
N+ stage			
N0	770 (58.6)	662 (64.5)	0.004
N+	544 (41.4)	364 (35.5)	
T stage			
T1	49 (3.7)	23 (2.2)	< 0.001
T2	377 (28.7)	121 (11.8)	
T3-4	888 (67.6)	882 (86.0)	

LNR: Lymph node ratio.

Number of positive lymph nodes, N stage and lymph node ratio between the two groups

The number of positive lymph nodes in the rectal carcinoma group was larger than that in the colon carcinoma group ($P < 0.001$). This was confirmed by comparison of N stage between the two groups. It was found that the patients in the rectal carcinoma group had a significantly higher N stage ($P < 0.001$) (Table 1).

The number of N+ patients in the rectal carcinoma group was significantly larger than that in the colon carcinoma group ($P = 0.004$) (Table 1). In addition, comparison of the lymph node ratio (LNR) of the stage Ⅲ cases between the two groups showed that LNR was higher in the rectal carcinoma group ($P < 0.001$).

Comparison of N+ stage stratified by T stage between the two groups

Advanced T stage was more likely to be associated with positive lymph nodes. A comparison of T stage between the two groups was undertaken to explore any possible impact of T stage on positive lymph node harvest. T3 and T4 tumors were taken together because most colon cancers were inherently located intraperitoneally, while more than half rectal cancers presented as extraperitoneal tumors. Considering the different definition of T3 and T4 stage of intraperitoneal and extraperitoneal tumors, it is more reasonable and comparable to put T3 and T4 tumors together to avoid location bias. It was noted that T stage was more advanced in the colon carcinoma group ($P < 0.001$). Moreover, Cochran's and Mantel-Haenszel statistics were used to compare patients with N+ stage between the two groups further stratified by T stage level. The N stage was significantly higher ($P < 0.001$) in the rectal carcinoma group, with an odds ratio (OR) of 1.617 (95% confidence interval: 1.355-1.931) (Table 2).

Table 2 Comparison between the percentage of patients with N+ stage

T stage[1]	N+ stage proportion (%)		*P* value
	Rectal cancer (*n* = 1314)	Colon cancer (*n* = 1026)	
T1	5/49 (10.2)	1/23 (4.3)	< 0.001
T2	84/377 (22.3)	23/121 (19.0)	
T3-4	455/888 (51.2)	340/882 (38.5)	

[1]Stratified by T stage.

DISCUSSION

Lymph node metastasis is an important prognostic factor for patients suffering from colorectal carcinoma without distant metastasis, as well as a risk factor for recurrence and distant metastasis of colorectal carcinoma[1-3]. The five-year survival rate of N+ patients was lower than that of N- patients[4]. In addition, the higher the number of positive lymph nodes examined, the poorer the prognosis would be. So in the colorectal TNM staging system[5,6], the node status was applied as the parameter to determine the stage Ⅲ tumors from the stage Ⅱ lesions, while the number of positive nodes indicated different N stages. In recent studies, LNR was recommended as a significant prognostic factor for both colon and rectal cancer patients[15,16], and thought to optimize staging in colorectal cancer[17]. Lymph node metastasis also affects patient management. It is generally accepted that adjuvant treatment is beneficial to N+ patients, but whether it is appropriate for N- patients remains controversial.

With respect to lymph node metastasis, colon cancer and rectal cancer were not often discussed independently[6,10,11]. Several studies compared the rates of lymph node metastasis between early rectal and colon cancers and found that they were different[12,13]. But the patient population in these studies was too small to draw a convincing conclusion. The present study explored possible differences in the lymph node metastasis pattern between colon and rectal carcinomas using strict inclusion criteria to eliminate patients who received neoadjuvant therapy, and those with stage Ⅳ tumors and incomplete information for the sake of minimizing influences of these factors on lymph node harvest. As several studies including our previous study had shown that neoadjuvant therapy decreased lymph node harvest and down-staged tumors[18], we excluded all patients who received neoadjuvant therapy in the present study. All procedures were performed by seven experienced colorectal surgeons with standard technique in one surgical section, and all specimens were treated in one pathology department to minimize interpretation variability.

In the present study, there was a higher percentage of

positive lymph node retrieval and N+ patients in the rectal carcinoma group in spite of a lowered total lymph node harvest. It is well established that T stage correlates with N stage[19,20], so T stage was investigated between the two groups to decrease background bias. Comparison of the overall lymph node status (N+) between the two groups based on T stage level also confirmed that there were more N+ patients in the rectal carcinoma group. Then the tumor size and grade were compared. Our data showed that the tumors in the rectal carcinoma group were smaller than those in the colon carcinoma group. In addition, there were more low-grade tumors in the rectal carcinoma group. Considering that high-grade big tumors harbored more metastatic lymph nodes[19,21], the primary tumor characteristics of the two groups enforced the hypothesis that rectal cancer is prone to metastasize to lymph nodes as compared with colon cancer. Furthermore, LNR of stage Ⅲ tumors was higher in the rectal carcinoma group than that in the colon carcinoma group.

Nevertheless, there were still some sources of bias in our retrospective study. The median number of total lymph nodes examined fell below 12, which would affect the accuracy of N stage. No long-term clinical follow-up was included, which may limit the ability to interpret the oncological significance of our findings. Further study is, therefore, required.

Different incidences of lymph node metastasis between colon and rectal cancers have several clinical implications. First, the minimal number required for lymph nodes retrieval remains controversial with a range from 7 to 21 in the literature[7-9]. Most of these studies combined colon and rectal cancers and the AJCC and College of American Pathologists recommend a minimum of 12 lymph nodes to be examined for both colon and rectal cancer[6,22]. In our study, the number of lymph nodes was significantly lower in the rectal cancer group compared with the colon cancer group. So the question arises whether the minimal number of lymph node harvest for colon and rectal cancer should be different. Second, the incidence of lymph node metastasis should be taken into account in choosing therapeutic strategies: a more aggressive strategy may be justified in treating rectal cancers than in colon cancers. As rectal carcinomas are more prone to metastasize to lymph nodes and the involved nodes might be the main reason of recurrence following local excision, radical resection rather than local excision seems to be a more reasonable recommendation for early rectal cancer as compared with colon cancer of similar primary tumor characteristics. For the same reason, adjuvant chemotherapy should be more suggestive for rectal cancer after local excision. Third, it is well known that the overall survival rate is lower and the local recurrence rate is higher in rectal caner following radical resection as compared with colon cancer. As we discussed earlier, the lymph node status is a key predictor for the prognosis of colorectal cancer. The differences seen in our study may explain the discrepancy in survival and local recurrence typically observed between colon and rectal cancers.

In conclusion, our study shows that the incidence of lymph node metastasis of rectal cancer is significantly higher than that of colon cancer as stratified by T stage, which may be attributed to different oncological and anatomic characteristics. This finding carries potential clinical significance related to treatment decision making, such as local excision or adjuvant therapy, as well as to prognostic assessment of rectal carcinoma.

COMMENTS

Background

Lymph node metastasis in colorectal cancer significantly influences patient management and prognosis. As to the issue of lymph node metastasis, colon cancers and rectal cancers are usually treated as a single entity.

Research frontiers

This paper essentially audits the colorectal cancer practice in one hospital over an 8-year period. The authors identified 2340 patients (1314 rectal, 1026 colonic) and retrospectively reviewed the pathological data related to these with particular respect to the number and distribution of lymph node metastases.

Applications

The rectal carcinoma seems more prone to metastasize to lymph nodes than colon carcinoma, which harbors potential clinical significance related to treatment decision making and prognosis interpretation.

Peer review

The authors analyzed the different patterns of lymph node metastasis in colon and rectal cancers. Although it is retrospective, the study involves a large patients' population over a relatively short period of time.

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BRIEF ARTICLE

STAT-3 correlates with lymph node metastasis and cell survival in gastric cancer

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Abstract

AIM: To investigate the correlation between gastric cancer growth and signal transducer and activator of transcription-3 (STAT3) expression.

METHODS: We assessed the expressions of STAT3, phosphor-STAT3 (pSTAT3), suppressor of cytokine signaling-1 (SOCS-1), survivin and Bcl-2 in gastric cancer patients after gastrectomy by immunohistochemical method. In addition, *in situ* hybridization was used to further demonstrate the mRNA expression of STAT3 in gastric cancer.

RESULTS: With the univariate analysis, expressions of STAT3, pSTAT3, SOCS-1, survivin and Bcl-2, the size of primary tumor and the lymph node metastasis were found to be associated with the overall survival (OS) of gastric cancer patients. However, only pSTAT3 expression and the lymph node metastasis were identified as the independent factors of OS of gastric cancer with multivariate analysis. STAT3 expression was correlated with the lymph node metastasis. There were positive correlations between expressions of STAT3, survivin, Bcl-2 and pSTAT3 in gastric cancer, whereas there was negative correlation between STAT3 expression and SOCS-1 expression in gastric cancer.

CONCLUSION: STAT3 can transform into pSTAT3 to promote the survival and inhibit the apoptosis of gastric cancer cells. SOCS-1 might be the valid molecular antagonist to inhibit the STAT3 expression in gastric cancer.

Key words: Gastric cancer; Signal transducer and activator of transcription-3; Lymph node metastasis; Apoptosis; Survival analysis

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INTRODUCTION

Gastric cancer is the fourth most common cancer worldwide with approximately 930 000 newly diagnosed cases annually, resulting in about 700 000 deaths per year[1,2]. Although the surgical skills and adjuvant chemotherapy have been improved, the survival rate of gastric cancer patients

remains unsatisfactory. Most gastric cancer patients were identified at the advanced stage when they were initially diagnosed, which is deemed as the important factor for the increased recurrence rate and decreased overall survival (OS) after potentially curative resection[3]. Therefore, it is imperative to investigate the molecular mechanism so as to enhance the curative effects for gastric cancer.

Many researchers have demonstrated that the aggressive nature of gastric cancer is related to mutations of various oncogenes and tumor suppressor genes and abnormalities in some growth factors and their receptors[4-6]. Recently, the activation of signal transducers and activators of transcription (STAT) proteins has been shown to have a strong bearing on gastric cancer progression although the detailed mechanism for this relationship has not been available[7]. The STAT proteins, including seven members, are a family of transcription factors which regulate expression of genes involved in both normal and pathological cellular processes. The strong association was demonstrated between STAT proteins and progression of various epithelial cancers[8-11].

Of the STAT family members, STAT3 is the most commonly activated in human cancers[12]. STAT3 is being increasingly recognized as a molecular hub for diverse signaling pathways such as cell cycle progression, apoptosis, angiogenesis and immune evasion, thus being considered as a novel molecular marker for cancer treatment[13,14]. Although STAT3 is constitutively activated and contributes to oncogenesis through preventing apoptosis and enhancing cell proliferation in many kinds of human tumors, the duration of STAT3 activation is temporary under physiologic conditions[15]. Over-expression of the activated STAT3 has been found in various types of malignant tumors, including leukemia, breast cancer. pancreatic cancer, prostatic cancer and melanoma, and STAT3 has played a key role in carcinogenesis[16]. However, the role of STAT3 signaling in gastric cancer is still unclear. Many studies suggested that the phospho-STAT3 (pSTAT3) should be taken as an active form of STAT3 and an independent prognostic factor for disease free survival and poor survival after curative resection[14,15].

Theoretically, STAT3 has to be activated by phosphorylated tyrosine induced by Janus Kinase (JAK) before STAT3 binds to receptor successfully. Subsequently, self-phosphorylated STAT3 protein is released from pSTAT3, assumes the shape of "dimmers" and migrates into the nucleus to activate the transcription of target genes. Jackson *et al*[17] found that pSTAT3 was localized mainly in epithelial cells in both normal stomach and gastric cancer, but there was at least ten-fold more STAT3 activation in the latter, with substantial nuclear staining. Therefore, a higher level of pSTAT3 positive expression represents more nuclear translocations of STAT3, which can stimulate the target gene transcription upon tissue transformation in gastric cancer. Suppressor of cytokine signaling-1 (SOCS-1) seems to be one of the STATs activated genes, which contain the SH2 domains that interact with JAK and prevent activation of STATs[18,19]. Recent findings suggest that SOCS-1 can be significantly up-regulated by interleukin (IL)-6 and is involved in the down-regulation of the IL-6-induced activation of STAT3[20]. Bcl-2 and survivin are the potential downstream molecules to STAT3. These molecules regulate both cell cycle and apoptosis and are the known targets of the STAT3 signaling pathway[21].

In the present study, we evaluated the expressions of STAT3, pSTAT3, SOCS-1, survivin, and Bcl-2 in both gastric cancer and normal gastric tissues after gastrectomy. We correlated these molecular variables, clinicopathologic features and prognoses of gastric cancer patients to study the potential mechanism of STAT3 signaling in carcinogenesis and progression of gastric cancer.

MATERIALS AND METHODS

Tissue specimens and patients

We used the human gastric cancer tissue and normal gastric tissue specimens preserved in the department of pathology and obtained patients' data from Tianjin Medical University Cancer Hospital. Fifty-three gastric cancer specimens from the 53 patients with gastric cancer and 53 normal gastric tissue specimens from 53 patients without gastric cancer were included in this study. Primary gastric cancer in these patients was diagnosed and treated at Tianjin Medical University Cancer Hospital between January 2002 and December 2002. The gastric cancer patients had well-documented clinical histories and follow-up information. None of them underwent preoperative chemotherapy and/or radiation therapy. All of gastric cancer patients had undergone potentially radical gastrectomy with lymphadenectomy. Of them, 37 (69.8%) were male and 16 (30.2%) were female. Ages ranged from 31 to 78 years, with a median age of 55.0 years. The tumor location was as follows: lower third in 28 (52.8%) cases, middle third in 18 (34.0%) cases, and upper third in 7 (13.2%) cases. Thirty-seven (69.8%) patients had lymph node metastasis identified by pathologic examination after surgery, and 16 (30.2%) patients had no lymph node metastasis after surgery. At the end of June 2009, 24 (45.3%) gastric cancer patients were still alive, whereas 29 (54.7%) had died. No gastric cancer patient died during the initial hospital stay or one month after surgery. Follow-up ranged from 4 to 85 mo, and the median was 35 mo. Results of B ultrasonography, CT scans, chest X-ray and endoscopy were obtained.

Methods

For *in situ* hybridization, the biotinylated oligonucleotide probe complementary to STAT3 (CCTTGGATTGAGAGTCAAGATTGGGCATAT) mRNA was synthesized. All *in situ* hybridization was carried out using manual capillary action technology. Briefly, the slides were rapidly deparaffinized, cleared, and rehydrated. The tissues were then digested with pepsin (Boshide, China) at 2 mg/mL for 30 min at 37℃. The probe was applied to the slides, and the tissues were heated at 42℃ for 2 h for denature and secondary mRNA structure. The hybridization of the probe and mRNA target was performed by exposing the slides in a calorstat at 42℃ for 12 h. The biotinylated

hybrids were detected with streptavidin-horseradish peroxidase (Jingmei, China) for 30 min at 37℃. After preincubation with 3,3'-diaminobenzidine solution for 3-5 min at 37℃, the tissues were washed with distilled water. Following the chromogen reaction, the tissues were counterstained with hematoxylin solution, washed with distilled water, air-dried, and cover-slipped with universal mount (Invitrogen US). *In situ* hybridization for negative control was performed with probe diluent.

Paraffin sections (4 μm thick) were deparaffinized and rehydrated. Antigen retrieval treatment was done at 95℃ for 40 min in 0.01 mol/L sodium citrate buffer (pH 6.0), and endogenous peroxidases were blocked using 3% hydrogen peroxide for 30 min. The primary antibodies at 1:100 dilution were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); STAT3 (SC-483, rabbit polyclonal IgG), SOCS-1 at 1:100 dilution (SC-9021, rabbit polyclonal IgG) from NeoMarkers Biotechnology, Inc. (Fremont, CA); survivin (RB-9245-P0, rabbit polyclonal IgG) at 1:50 dilution, and Bcl-2 (Ms-123-PABX, mouse monoclonal IgG) at 1:100 dilution from Jingmei Biotechnology (Zhongshan, China). All sections were incubated overnight with the primary antibody at 4℃. The sections were then treated with peroxidase by the labeled polymer method with Zhongshan peroxidase for 30 min. Antibody binding was visualized using the Avidin Biotin Complex Elite Kit and 3,3-diaminobenzine according to the manufacturer's instructions (City Key Laboratory of Tianjin Cancer Center, China). Sections were then counterstained in hematoxylin. Breast cancer and colon cancer specimens confirmed to be immunoreactive for the relevant antigens in preliminary studies were used as positive controls for STAT3, pSTAT3, SOCS-1, survivin and Bcl-2. For general negative controls, the primary antibody was replaced with phosphate buffered saline.

All sections were scored blindly by two independent observers, and in cases of scoring disagreement, a third independent assessment was performed. Staining of STAT3, SOCS-1, survivin, and Bcl-2 was graded according to the intensity and extent of staining of the epithelium. The staining intensity was scored into 4 grades: 0, complete absence of the staining; 1, weak staining; 2, moderate; and 3, strong staining. The extent of the positively stained cells was also scored into 5 grades: 0, a complete negative staining; 1, < 5%; 2, 5%-25%; 3, 25%-75%; and 4, ≥ 75%. The final scores were derived from multiplication of the extent by the intensity. For statistical analysis, scores were further grouped into two categories: negative (final scores, < 3) and positive (final score, ≥ 3).

The staining of pSTAT3 was grouped as positive and negative. The positive staining of pSTAT3 was defined as ≥ 10% of the cells with the nucleus stained dark brown and the negative staining was defined as < 10% of the cells stained.

Statistical analysis

All statistical calculations were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL). Differences in STAT3 expression, pSTAT3 expression, SOCS-1 expression, surviving expression, Bcl-2 expression and clinicopathologic variables of patients were estimated using the χ^2 test for categorical data and independent-paired Student's t test for continuous variables. Overall survival of gastric cancer patients was measured from date of surgery to the date of death by the Kaplan-Meier method. The log-rank test (χ^2 comparison) was used to compare the overall survival based on expressions of STAT3, pSTAT3, SOCS-1, survivin and Bcl-2. Cox regression analysis was used to estimate the independent risk factors of overall survival of gastric cancer patients after radical surgery. In addition, linear regression was used to estimate the correlations among the variables which were significantly associated with the STAT3 immunohistochemical expression.

RESULTS

Expression of STAT3 mRNA by in situ hybridization

Almost all gastric cancer tissue specimens were positive for STAT3 mRNA expression, whereas only 8 of 53 normal gastric tissue specimens were positive for STAT3. STAT3 mRNA expression was strong in the cytoplasm of gastric cancer epithelium, while the epithelium of normal gastric tissues was weak or negative (Figure 1A).

Immunohistochemical differences of STAT3, pSTAT3, SOCS-1, survivin, and Bcl-2 between gastric cancer tissue and normal gastric tissue specimens

STAT3 was detected mainly in the epithelium of gastric cancer tissues and less frequently in the normal gastric tissues. The strong granular patterns of STAT3 staining were observed mainly within the cytoplasm and partially within the nuclear of the epithelium of gastric cancer. Conversely, only weak granular patterns of STAT3 staining were observed within the cytoplasm of a few normal gastric tissue specimens (Figure 1B). STAT3 staining was quantitatively assessed and grouped into positive expression and negative expression. Positive STAT3 expression was detected in 31 of 53 gastric cancer tissue specimens and 6 of 53 normal gastric tissue specimens. The ratio of positive STAT3 expression in gastric cancer tissue specimens was significantly higher than that in normal gastric tissue specimens (58.5% *vs* 11.3%, $\chi^2 = 25.950$, $P < 0.001$).

pSTAT3 was detected mainly in the epithelium of gastric cancer tissues and less frequently in the normal gastric tissues. The strong granular patterns of pSTAT3 staining were observed mainly within the nuclear and partially within the cytoplasm of the epithelium of gastric cancer. Conversely, only weak granular patterns of pSTAT3 staining were observed within the nuclear of a few normal gastric tissue specimens (Figure 1C). pSTAT3 staining was quantitatively assessed and grouped into positive expression and negative expression. Positive pSTAT3 expression was detected in 26 of 53 gastric cancer tissue specimens and 2 of 53 normal gastric tissue specimens. The ratio of positive pSTAT3 expression in gastric cancer tissue specimens was significantly higher than that in normal gastric tissue specimens (49.1% *vs* 3.8%, $\chi^2 = 27.956$, $P < 0.001$).

SOCS-1 was detected mainly in the normal gastric

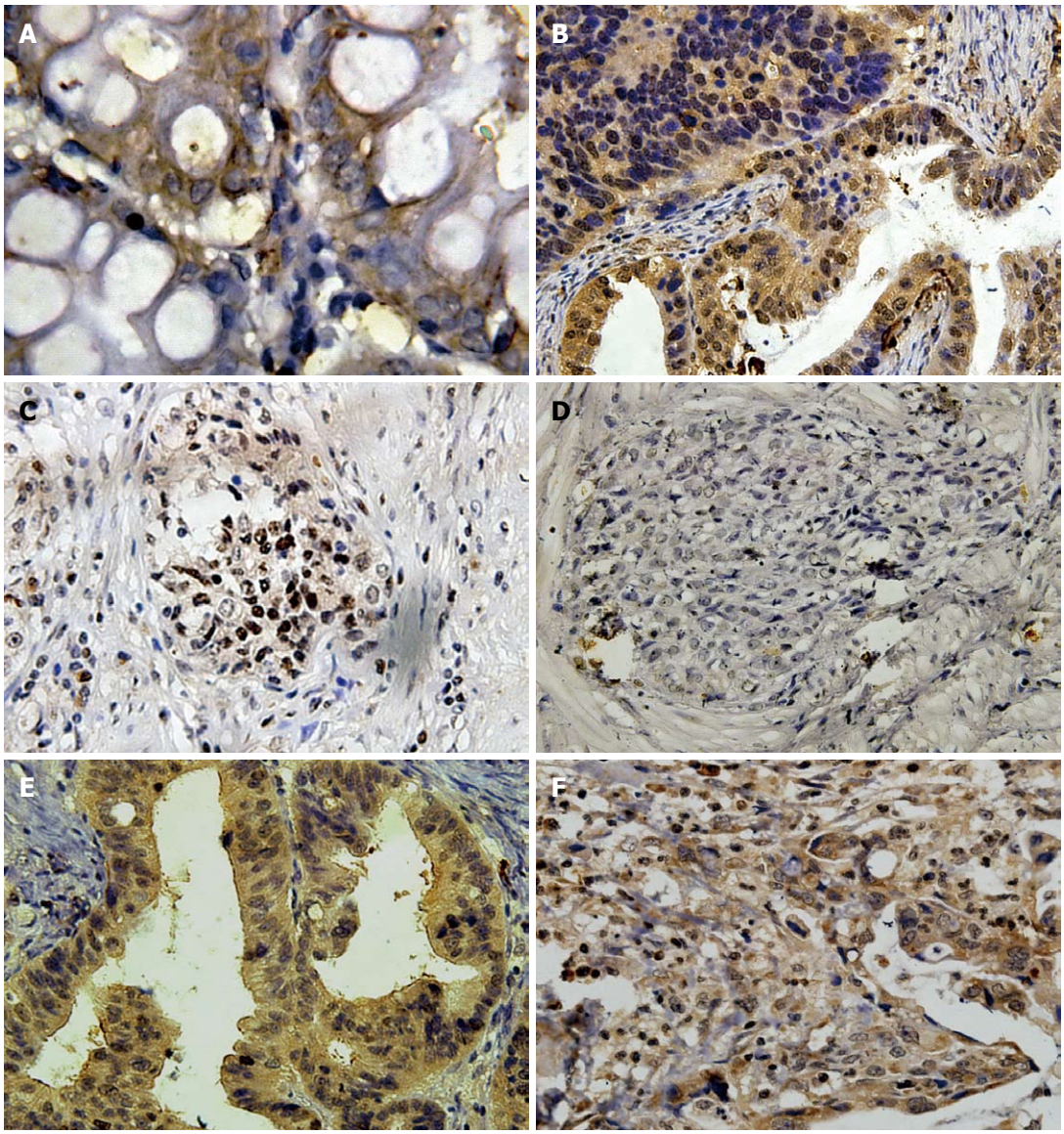

Figure 1 Expression in the cytoplasm of gastric cancer tissues (× 400). A: Detection of signal transducer and activator of transcription-3 (STAT3) mRNA (*in situ* hybridization); B: STAT3 (IH); C: Phosphor-STAT3 (IH); D: Suppressor of cytokine signaling-1 (IH); E: Survivin (IH); F: Bcl-2 (IH).

tissues and less frequently in the epithelium of gastric cancer. The strong granular patterns of SOCS-1 staining were observed within the cytoplasm of the normal gastric tissue specimens. Conversely, only weak granular patterns of SOCS-1 staining were observed within the cytoplasm of a few gastric cancer tissue specimens (Figure 1D). SOCS-1 staining was quantitatively assessed and grouped into positive expression and negative expression. Positive SOCS-1 expression was detected in 6 of 53 gastric cancer tissue specimens and 23 of 53 normal gastric tissue specimens. The ratio of positive STAT3 expression in gastric cancer tissue specimens was significantly lower than that in normal gastric tissue specimens (11.3% *vs* 43.4%, χ^2 = 13.719, $P < 0.001$).

Survivin was detected mainly in the epithelium of gastric cancer tissues and less frequently in the normal gastric tissues. The strong granular patterns of survivin staining were observed within the cytoplasm of the epithelium of gastric cancer tissue specimens. Conversely, only weak granular patterns of survivin staining were observed within the cytoplasm of a few normal gastric tissue specimens (Figure 1E). Survivin staining was quantitatively assessed and grouped into positive expression grade and negative expression. Positive survivin expression was detected in 39 of 53 gastric cancer tissue specimens and 4 of 53 normal gastric tissue specimens. The ratio of positive survivin expression in gastric cancer tissue specimens was significantly higher than that in normal gastric tissue specimens (73.6% *vs* 7.5%, χ^2 = 47.933, $P < 0.001$).

Bcl-2 was detected mainly in the epithelium of gastric cancer tissues and less frequently in the normal gastric tissues. The strong granular patterns of Bcl-2 staining were observed within the cytoplasm of the malignant epithelium of gastric cancer tissue specimens. Conversely, only weak granular patterns of Bcl-2 staining were observed within the cytoplasm of a few normal gastric tissue specimens (Figure 1F). Bcl-2 staining was quantitatively assessed and grouped into positive expression and negative

Table 1 Univariate analysis of factors affecting overall survival of 53 gastric cancer patients by Kaplan - Meier method

Factor	*n*	5-yr survival rate (%)	χ^2	*P* value
Gender			0.217	0.641
Male	37	45.9		
Female	16	43.8		
Age at surgery (yr)			0.260	0.610
< 65	40	42.5		
⩾ 65	13	53.8		
Tumor size (cm)			5.711	0.017
< 4	13	76.9		
⩾ 4	40	35.0		
Tumor location			2.619	0.270
Lower stomach	28	57.1		
Middle stomach	18	33.3		
Upper stomach	7	28.6		
Depth of primary tumor invasion			3.089	0.079
T1 + T2	6	83.3		
T3 + T4	47	40.4		
Status of lymph node metastasis			17.950	< 0.001
Negative	16	93.8		
Positive	37	24.3		
Lauren's classification			3.280	0.070
Intestinal	25	60.0		
Diffuse	28	32.1		
STAT3			19.432	< 0.001
Negative	22	81.8		
Positive	31	19.4		
pSTAT3			40.444	< 0.001
Negative	27	85.2		
Positive	26	3.8		
SOCS-1			5.712	0.017
Negative	47	38.3		
Positive	6	100.0		
Survivin			4.295	0.038
Negative	14	71.4		
Positive	39	35.9		
Bcl-2			13.866	< 0.001
Negative	27	70.4		
Positive	26	19.2		

STAT3: Signal transducer and activator of transcription-3; pSTAT3: Phosphor-STAT3; SOCS-1: Suppressor of cytokine signaling-1.

expression. Positive Bcl-2 expression was detected in 26 of 53 gastric cancer tissue specimens and in 0 of 53 normal gastric tissue specimens. The ratio of positive Bcl-2 expression in gastric cancer tissue specimens was significantly higher than that in normal gastric tissue specimens (49.1% *vs* 0%, $\chi^2 = 34.450$, $P < 0.001$).

Analysis of clinicopathologic variables and immunohistochemical staining of antibodies in gastric cancer

The following clinicopathologic variables were analyzed: (1) age at surgery (< 65 years or ⩾ 65 years); (2) gender (male or female); (3) tumor location (lower third, middle third, or upper third); (4) tumor size (< 4 cm or ⩾ 4 cm); (5) depth of primary tumor invasion (UICC) (T1+T2, or T3+T4); (6) Lauren's classification of primary tumor (intestinal or diffuse); and (7) metastatic status of lymph nodes (positive or negative). In addition, the following antibodies were analyzed with OS of gastric cancer patients: STAT3, pSTAT3, SOCS-1, survivin and Bcl-2.

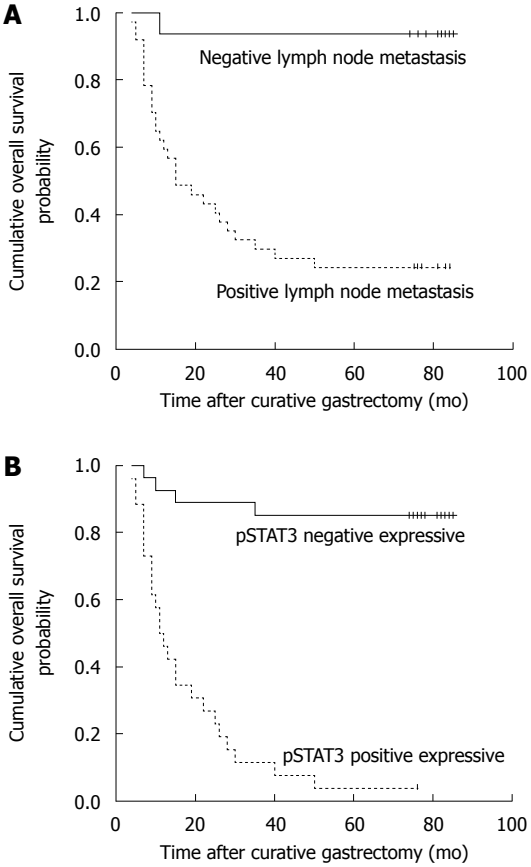

Figure 2 Survival curve in 53 gastric cancer patients following curative resection according to the metastatic status of lymph nodes (A) and different expression levels of phosphor-signal transducer and activator of transcription-3 (B). A: Negative lymph node metastasis and positive lymph node metastasis; B: Negative and positive expression of pSTAT3.

With the univariate analysis, seven factors were found to have statistically significant associations with OS of gastric cancer patients after curative surgery (Table 1), including tumor size, metastatic status of lymph nodes (Figure 2A), and STAT3, pSTAT3 (Figure 2B), SOCS-1, survivin and Bcl-2 expressions.

All above seven variables were included in a multivariate Cox proportional hazards model (forward stepwise procedure) to adjust for the effects of covariates (Table 2). In this model, metastatic status of lymph nodes (HR = 8.591, $P = 0.040$), and pSTAT3 (HR = 9.605, $P < 0.001$) showed significant correlations with OS of gastric cancer patients after curative surgery.

Correlation between immunohistochemical expressions of STAT3, pSTAT3, SOCS-1, survivin and Bcl-2 and status of lymph node metastasis in gastric cancer

We found that the immunohistochemical expression level of STAT3 was positively associated with that of pSTAT3 ($\beta = 0.451$, $P = 0.001$), survivin ($\beta = 0.504$, $P = 0.001$), and Bcl-2 ($\beta = 0.588$, $P < 0.001$). The immunohistochemical expression level of STAT3 was negatively associated with that of SOCS-1 ($\beta = -0.660$, $P = 0.002$). In addition, the STAT3 immunohistochemical expression was positively associated with the status of lymph node metastasis ($\beta = 0.480$, $P = 0.001$).

Table 2 Multivariate analysis of factors affecting overall survival of 53 gastric cancer patients by Cox proportional hazards model

Factors	*P* value	Relative risk	95% CI
Status of lymph node metastasis	0.040	8.591	1.104-66.858
Tumor size	0.701		
STAT3	0.225		
pSTAT3	< 0.001	9.605	3.107-29.694
SOCS-1	0.429		
Survivin	0.749		
Bcl-2	0.213		

STAT3: Signal transducer and activator of transcription-3; pSTAT3: Phosphor-STAT3; SOCS-1: Suppressor of cytokine signaling-1; CI: Confidence interval.

Correlation between immunohistochemical expressions of pSTAT3, SOCS-1, survivin and Bcl-2 and status of lymph node metastasis in gastric cancer

The immunohistochemical expression level of pSTAT3 was positively associated with that of survivin ($\beta = 0.473$, $P = 0.002$) and Bcl-2 ($\beta = 0.623$, $P < 0.001$). The immunohistochemical expression level of pSTAT3 was negatively associated with that of SOCS-1 ($\beta = -0.553$, $P = 0.010$). In addition, the level of pSTAT3 immunohistochemical expression was positively associated with the status of lymph node metastasis ($\beta = 0.524$, $P < 0.001$).

DISCUSSION

Recently, the molecular markers are being studied, as it may improve the prognostic prediction for gastric cancer patients after curative resection. Although many investigators have suggested that several molecular markers should be considered as key prognostic indicators in gastric cancer, such as epidermal growth factor receptor, vascular endothelial growth factor, hypoxia inducible factor 1-α and p53, none of them have been defined as a unified standard in clinical practice[22-24]. The STAT proteins are a family of transcription factors which regulate expression of multiple genes involved in both physiological and pathological conditions, which seems to be one of the most promising molecular markers for predicting the prognosis of patients with various cancers.

Of the STAT family members, STAT3 is the most commonly activated in human cancers[12]. Several previous reports showed increased or activated STAT3 expression in gastric cancer[15,16,25]. Recently, Kim *et al*[7] found that 39% of 100 patients with gastric cancer had positive STAT3 expression, whereas only 14% of 71 patients with normal gastric tissues had positive STAT3 expression. They also demonstrated that positive STAT3 expression was significantly associated with regional lymph node invasion ($P = 0.008$). Jackson *et al*[17] indicated that inappropriate STAT3 activation was an early event in the initiation and progression of gastric cancer. They found that gastric cancer cells showed increased total STAT3 staining in both the cytoplasm and the nucleus, and a high degree of predominantly nuclear pSTAT3 staining as compared with normal gastric cells. At present study, the ratio of positive STAT3 expression in gastric cancer tissue specimens was 58.5%, which was significantly higher than that in normal gastric tissue specimens (11.3%) ($P < 0.001$). Similarly, our results also showed that positive STAT3 expression was significantly associated with the status of lymph node metastasis ($P = 0.001$). Our positive STAT3 expression was higher than that reported by Kim *et al*[7], and lower than that (73%) reported by Gong *et al*[16]. We think these differences might be induced due to the different stages of patients, subjective interpretation by pathologists, and production of antibodies. To avoid the limitation of STAT3 expression detected by the immunohistochemical method, we also adopted the *in situ* hybridization for a more precise detection of STAT3 mRNA expression. All gastric cancer tissue specimens were positive for STAT3 mRNA expression, whereas only 8 of 53 normal gastric tissue specimens were positive for STAT3. With the survival analysis, we found that patients with STAT3 negative expression in primary tumors were more likely to have a longer median OS compared with those with STAT3 positive expression ($P < 0.001$). This result was similar to the above-mentioned studies. Therefore, we thought that the STAT3 positive expression was significantly associated with the progression and prognosis of gastric cancer patients.

Choi *et al*[26] analyzed 137 cases of gastric cancer tissues obtained from radical gastrectomy and found that pSTAT3 positive expression occurred more frequently than in non-neoplastic gastric tissues and pSTAT3 positive expression was significantly associated with low pathological grade and lymph node metastasis. Lee *et al*[14] assessed the STAT3 activation in 311 cases of surgically resected gastric cancer tissues and found that pSTAT3 was an independent prognostic factor for poor survival following curative resection. We also found that pSTAT3 positive expression in gastric cancer tissues occurred significantly more often than that in normal gastric tissues and pSTAT3 was significantly associated with the status of lymph node metastasis. The survival analysis showed that pSTAT3 was an independent factor of OS after curative resection in gastric cancer patients instead of STAT3. Therefore, pSTAT3 might be a functional transformant which could preferably reflect the biological effects of STAT3.

Many studies demonstrated that the inactivated SOCS-1 was one of the targets in cancer development and a tumor suppressor in the JAK/STAT pathway[27-29]. Ni *et al*[30] found that there was down-regulation of SOCS-1 gene in gastric cancer cell line AGS due to gene promoter hypermethylation, which provided evidence that JAK/STAT pathway was activated by aberrant SOCS-1 methylation in gastric cancer. In our study, the SOCS-1 positive expression in gastric cancer was much lower than that in normal gastric tissues ($P < 0.001$). Besides, the SOCS-1 expression was negatively correlated with the expression of STAT3 ($P = 0.002$) or pSTAT3 ($P = 0.010$). We considered that SOCS-1 was an inhibitor for the expression, activation and phosphorylation of STAT3 in gastric cancer.

STAT3 has been considered as a molecular hub for several crucial signaling pathways in different cancer types. It was suggested that the activation of cell proliferation and inhibition of apoptosis were induced by STAT3[30]. Several researchers showed the similar conclusion that oncogenesis of the STAT3 was closely associated with the cell survival and the cell apoptosis[15,25,31]. In our study, the ratio of positive Bcl-2 expression in gastric cancer tissues was significantly higher than that in normal gastric tissues and the Bcl-2 expression was significantly associated with STAT3 (or pSTAT3) expression. Similar results were found in survivin expression. Therefore, we considered that STAT3 could facilitate the progression of gastric cancer by supporting cell survival and inhibiting cell apoptosis.

In summary, the STAT3 expression was significantly associated with the lymph node status, poor prognosis, and the expressions of pSTAT3, survivin and Bcl-2. The SOCS-1 expression was inversely associated with the STAT3 expression. STAT3 could promote tumor cell survival and inhibit cell apoptosis in gastric cancer.

COMMENTS

Background

Signal transducer and activator of transcription-3 (STAT3) was identified as the key factor which was associated with cancers by many investigators. However, the correlation between gastric cancer and STAT-3 is not unclear.

Research frontiers

The authors detected the expressions of STAT3, phosphor-STAT3 (pSTAT3), SOCS-1, survivin and Bcl-2 proteins in gastric cancer and analyzed the clinicopathological variables associated with the prognosis of gastric cancer. Multivariate analysis showed that pSTAT3 expression and lymph node metastasis were independent factors of overall survival of gastric cancer patients. The expression of STAT3 protein was associated with the status of lymph node metastasis of gastric cancer.

Innovations and breakthroughs

STAT3 could transform into pSTAT3 to promote the survival and inhibit the apoptosis of gastric cancer cells. suppressor of cytokine signaling-1 (SOCS-1) might be the valid molecular antagonist to inhibit the STAT3 expression in gastric cancer.

Applications

The molecular contributions of STAT3 to gastric cancer have been elucidated in this study.

Terminology

STAT3 is significantly associated with the lymph node metastasis, cell survival and inhibition of cell apoptosis in gastric cancer.

Peer review

Deng *et al* studied the expressions of STAT3, pSTAT3, SOCS-1, survivin and Bcl-2 proteins in human gastric cancer. The study is of interest and potential clinical importance.

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CASE REPORT

Modified rendezvous intrahepatic bile duct cannulation technique to pass a PTBD catheter in ERCP

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Abstract

The rendezvous procedure combines an endoscopic technique with percutaneous transhepatic biliary drainage (PTBD). When a selective common bile duct cannulation fails, PTBD allows successful drainage and retrograde access for subsequent rendezvous techniques. Traditionally, rendezvous procedures such as the PTBD-assisted over-the-wire cannulation method, or the parallel cannulation technique, may be available when a bile duct cannot be selectively cannulated. When selective intrahepatic bile duct (IHD) cannulation fails, this modified rendezvous technique may be a feasible alternative. We report the case of a modified rendezvous technique, in which the guidewire was retrogradely passed into the IHD through the C2 catheter after end-to-end contact between the tips of the sphincterotome and the C2 catheter at the ampulla's orifice, in a 39-year-old man who had been diagnosed with gallbladder carcinoma with a metastatic right IHD obstruction. Clinically this procedure may be a feasible and timesaving technique.

Key words: Endoscopic retrograde cholangiopancreatography; Intrahepatic bile duct; Rendezvous technique

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INTRODUCTION

Selective deep cannulation of the bile duct is essential for the successful treatment of biliary obstructions. However, even the most experienced endoscopists fail to cannulate the biliary tract in 5%-10% of cases[1-4]. When a selective biliary cannulation fails, percutaneous transhepatic biliary drainage (PTBD) allows successful drainage and retrograde access for subsequent rendezvous techniques. Traditionally, rendezvous procedures such as the PTBD-assisted over-the-wire cannulation method, the parallel cannulation technique, or others may be available when a bile duct cannot be selectively cannulated[5-9]. When a selective intrahepatic bile duct (IHD) cannulation fails, a rendezvous technique may be an alternative.

We describe a modified rendezvous technique in a case of right IHD obstruction caused by metastatic gallbladder malignancy. After end-to-end contact between the tips of the sphincterotome and the C2 catheter at the ampulla's orifice, the guidewire was retrogradely passed into the IHD through the C2 catheter. Clinically this procedure is feasible and timesaving. To the best of our knowledge,

this technique has not yet appeared in the English language literature.

CASE REPORT

A 39-year-old man with no specific medical history presented with right upper quadrant abdominal pain and weight loss that had started to worsen a month before presentation. Laboratory tests revealed the following: total bilirubin, 28 mg/L (normal 2-12 mg/L); aspartate transaminase/alanine transaminase, 132/331 IU/L (normal 0-40 IU/L); alkaline phosphatase, 497 IU/L (normal 39-117 IU/L); γ-glutamyltransferase, 571 IU/L (normal 0-52 IU/L); and CA19-9, 56.3 U/mL (normal 0-34 U/mL). Abdominal computed tomography revealed diffuse low-attenuated wall thickening of the gallbladder with multiple metastatic nodules on the subhepatic and right-lower abdomen. Magnetic resonance cholangiopancreatography images showed severe segmental stricture of the right IHD with mild peripheral ductal dilatation (Figure 1).

Subsequent endoscopic retrograde cholangiopancreatography (ERCP) showed that the contrast did not pass through. Selective cannulation of the right IHD was impossible even using various sized guidewires and papillotomes such as Autotome (Boston Scientific, Microvasive, Marlboro, MA, USA) because of severe obstruction (Figure 2). Access to the strictured IHD was obtained by PTBD using a 5 F, 75 cm C2 catheter (Cook Endoscopy, Winston-Salem, NC, USA). After PTBD, a C2 catheter was placed into the duodenum through the stricture site and CBD (Figure 3). In the second ERCP, a rendezvous procedure was planned to selectively access the right IHD. A guidewire-preloaded sphincterotome was positioned onto the ampulla and the C2 catheter was then slowly pulled out near the ampulla's orifice. After end-to-end contact between the tips of the sphincterotome and the C2 catheter at the ampulla's orifice, the guidewire was passed into the IHD through the C2 catheter, while simultaneously, the sphincterotome was pushed and the C2 catheter was pulled out (Figures 3 and 4). The guidewire was then selectively placed into the IHD. Finally, following balloon dilatation (Hurricane balloon, 4 mm, 60 s; Boston Scientific, MA, USA), a 7 F Amsterdam stent (Cook Endoscopy) was successfully inserted into the IHD. However, endoscopic biopsy or cytology on the stricture site was not performed. A ultrasonography-guided biopsy of the metastatic mass in the right-lower abdomen diagnosed it as an adenocarcinoma (immunohistochemical stains; CK7+, CK20-, and CDX2-). The patient was placed on concurrent systemic chemotherapy and radiotherapy.

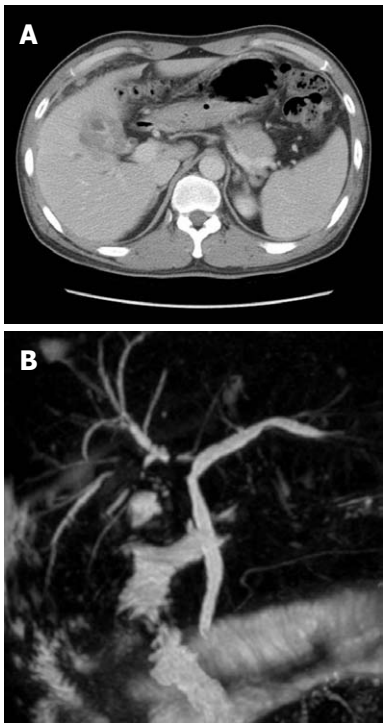

Figure 1 Abdominal computed tomography. A: Diffuse low-attenuated wall thickening of the gallbladder; B: Magnetic resonance cholangiopancreatography reveals a severe segmental stricture of the right intrahepatic bile duct with mild upstream duct dilatation.

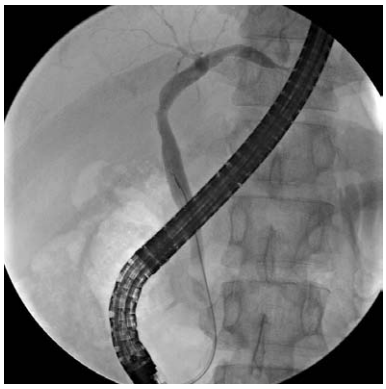

Figure 2 Endoscopic retrograde cholangiopancreatography shows severe narrowing of the right proximal intrahepatic bile duct and slight narrowing of the common hepatic duct.

DISCUSSION

When a selective CBD cannulation cannot be performed at ERCP despite trying various endoscopic techniques, PTBD followed by a combined rendezvous technique is often successful[5-13]. This combined technique increases the success rate of biliary tract cannulation and facilitates the diagnosis and treatment of biliary tract diseases. Initially, a PTBD-placed guidewire is grasped at the tip of the duodenoscope; then the duodenoscope and wire are withdrawn[8]. This technique has some limitations, such as the two sessions required for the endoscopic procedure, difficulty grasping the wire with forceps, and kinking of the guidewire. Mönkemüller *et al*[9] recommend advancing the guidewire into the catheter's tip so that it can guide cannulation of the ampulla, but this can be difficult and requires that the wire be passed carefully. Dickey suggested a parallel cannulation technique using a sphincterotome retrogradely, alongside a biliary drainage catheter[7]. This technique can be performed in one session if CBD cannulation is difficult.

However, even if selective CBD cannulation is successful, when a selective IHD cannulation fails, this modi-

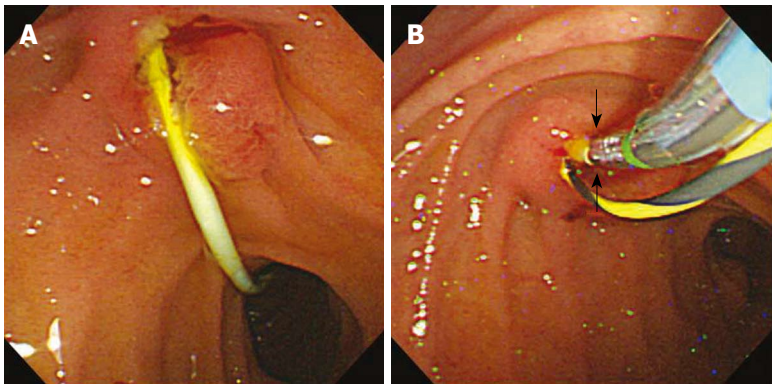

Figure 3 Endoscopic images. A: A C2 catheter in the duodenum; B: A view of end-to-end contact between the tips of the guidewire-preloaded sphincterotome and C2 catheter (arrows) at the ampulla's orifice.

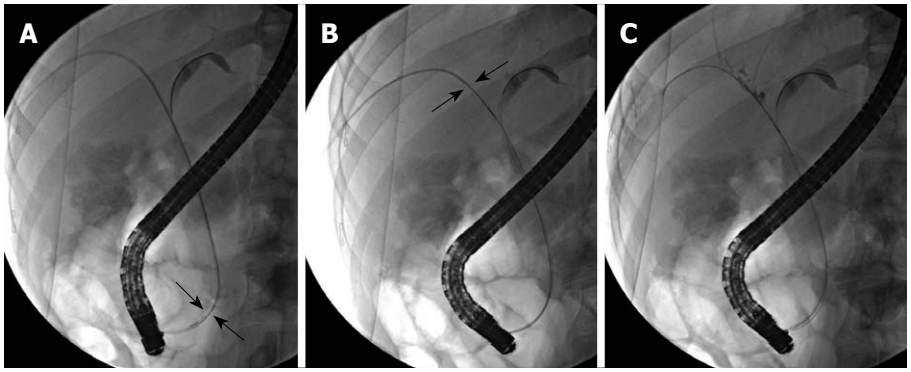

Figure 4 Fluoroscopic views. A: The guidewire passed into the right intrahepatic bile duct through the C2 catheter after end-to-end contact (arrows); B: Simultaneously, the sphincterotome was pushed into the common bile duct and the C2 catheter was pulled out (arrows); C: Finally, the C2 catheter was removed.

fied rendezvous technique may be useful. In our case, the right IHD was almost totally obstructed due to direct invasion of a gallbladder carcinoma, which prevented selective IHD cannulation despite various attempts at cannulation. Moreover, even though PTBD was successful, a 5 F C2 catheter, rather than a 7-10 F catheter which is commonly used, was placed because of severe stricture and abdominal pain during the procedure. The modified rendezvous procedure described above was then performed successfully. In cases of tight bile duct obstruction without marked ductal dilatation, it is difficult to place a metal stent *via* a PTBD route. In addition, plastic stent insertion is not possible *via* PTBD. The size of the transhepatic track necessary to insert a plastic stent percutaneously is related to the high complication rates of percutaneous stenting[14]. Endoscopic retrograde access was also impossible because of the inserted PTBD catheter. In such cases, this rendezvous technique can be a viable alternative.

In conclusion, this modified rendezvous technique for selective IHD cannulation appears to be a safe and feasible procedure. This technique is useful in selected cases such as the one presented here and in cases of failed selective CBD cannulation.

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CASE REPORT

Achalasia combined with esophageal intramural hematoma: Case report and literature review

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Abstract

A 62-year-old male patient was admitted to our hospital due to severe chest pain, odynophagia, and hematemesis. Chest computed tomography showed an esophageal submucosal tumor. Esophagogastroduodenoscopy (EGD) revealed a longitudinal purplish bulging tumor of the esophagus. Endoscopic ultrasound (EUS) showed a mixed echoic tumor with partial liquefaction from the submucosal layer. The patient was diagnosed with esophageal intramural hematoma as well as achalasia by upper gastrointestinal endoscopy, esophagography and esophageal manometry. The patient was managed conservatively with intravenous nutrition, and oral feeding was discontinued. Follow-up EGD and EUS showed complete recovery of the esophageal wall, and finally, the patient underwent endoscopic dilatation for achalasia. The patient was symptom free at the time when we wrote this manuscript.

Key words: Esophageal intramural hematoma; Achalasia; Endoscopic ultrasound

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INTRODUCTION

Esophageal intramural hematoma (EIH) is an uncommon cause of severe chest pain. More than 63% of patients with EIH have an underlying etiology or predisposing factors such as esophageal instrumentation, vomiting, trauma, pill-induced injury, food impaction-related issue, or coagulation defects[1]. We report a patient with severe chest pain, dysphagia, and upper gastrointestinal bleeding who was subsequently diagnosed with EIH and achalasia. The patient was initially diagnosed by esophagogastroduodenoscopy (EGD) and underwent endoscopic ultrasound (EUS) for further confirmation. After conservative treatment, the patient recovered well and then underwent esophageal manometric examination and a series of EGD and EUS follow-up.

CASE REPORT

A 62-year-old male patient had a history of hypertension

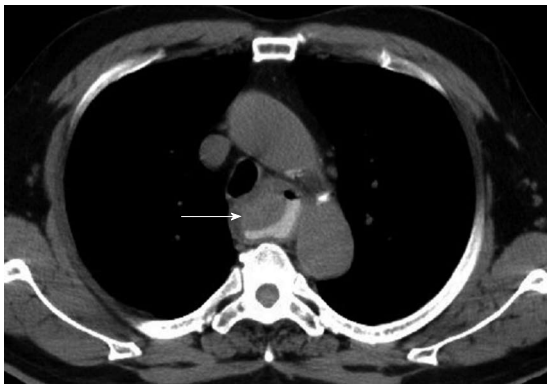

Figure 1 Computed tomography revealing a slight hyperdense tumor lesion (arrow) with a smooth surface over the upper esophagus, and no false lumen.

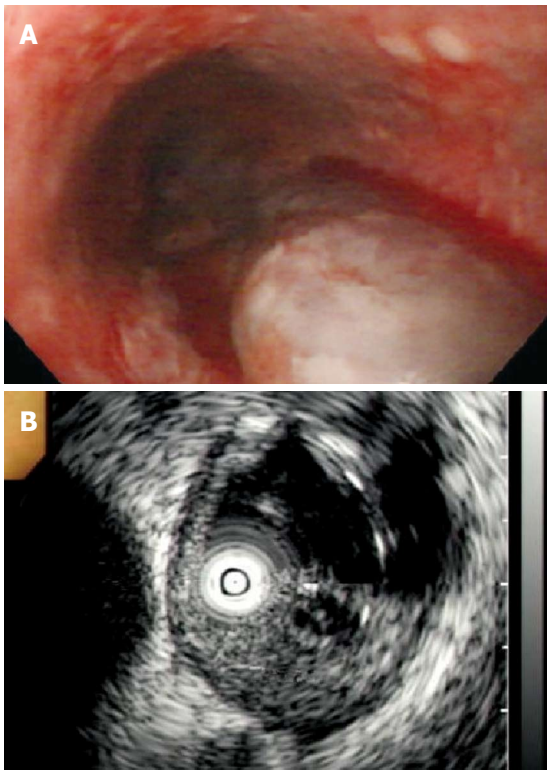

Figure 2 Esophagogastroduodenoscopy revealing a purplish bleeding esophageal submucosal tumor (A) and endoscopic ultrasound revealing a mixed echoic tumor with an anechoic component and an irregular at the submucosal layer (B).

which was treated with regular calcium channel antagonists. He suffered from dysphagia, intermittent anterior chest pain, and poor oral intake for 1 mo but no nausea or vomiting, no acid or food regurgitation, and no significant body weight loss. He did not seek medical aid until he experienced an episode of severe anterior chest pain, odynophagia, and hematemesis. He was sent to the emergency room. Laboratory results revealed a hemoglobin level of 13.5 g/dL and a platelet count of 205000/mL. Electrocardiography showed sinus tachycardia, and chest X-ray was normal. Chest computed tomography revealed a slight hyperdense tumor with a smooth surface over the upper thoracic esophagus anterior wall extending to the subcarina area with no evidence of aortic dissection (Figure 1). He then received EGD which revealed a longitudinal oozing purplish, bulging tumor from the upper to mid-esophagus. Differential diagnosis included metastatic malignancy, submucosal tumor, and hematoma. EUS showed a mixed echoic and partially liquefied mass lesion with irregular margins over the submucosal layer (Figure 2). Intramural hematoma of the esophagus was diagnosed based on these examinations. Two days after the patient was managed as nil by mouth, through use of intravenous nutrition and analgesics, he received a follow-up EGD, which revealed a dissected necrotic mass lesion with submucosal ulceration (Figure 3). Endoscopic biopsy was performed to exclude malignancy, and pathology showed necrosis and acute inflammation but no malignant cells. Follow-up EGD performed 1 wk later revealed complete resolution of the submucosal hematoma with longitudinal superficial ulcer, and EUS showed no mass lesions and only mild thickening of the mucosal and submucosal layer (Figure 4). The patient began to try liquid diet intake and was discharged on the 10th day after admission. One month later, during an outpatient visit, the patient still had dysphagia symptoms. EGD showed an intact esophageal mucosa with a tight esophagocardiac junction (ECJ) and residual food accumulation in the esophagus. Barium esophagography showed a normal esophageal wall contour but a dilated lumen with narrowing of the ECJ (Figure 5), and achalasia was suspected. The patient also received an esophageal manometric examination, which revealed 90% simultaneous contraction of the esophageal body, impaired relaxation of the low esophageal sphincter (LES) and LES pressure of 58 mm Hg. These findings were compatible with achalasia, so he received endoscopic dilatation twice for achalasia. After treatment, the patient returned to normal oral intake and symptom free during the 18-mo follow up.

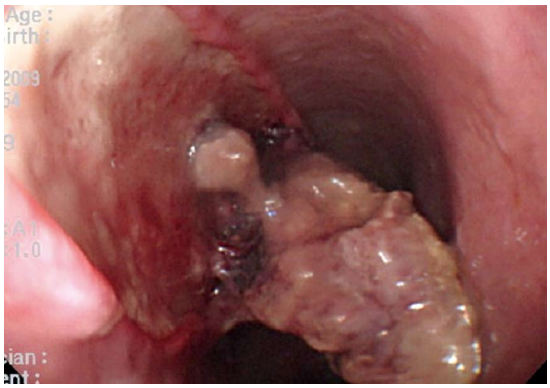

Figure 3 Follow-up esophagogastroduodenoscopy 2 d later revealing a dissected necrotic mass lesion with ulcer formation.

DISCUSSION

EIH is a rare disease that was first described by Williams in 1957[2]. Most reported cases of EIH occurred in women. The age range of patients with EIH is 20-89 years,

Table 1 Reports on achalasia in patients with esophageal intramural hematoma[8-11]

Authors	Age (yr)	Sex	Interval between the diagnosis of achalasia and EIH	Treatment of achalasia	EIH recurrence (Interval between recurrence)
Freeman *et al*[8]	69	F	3 mo[1]	Surgery	No
Herbetko *et al*[9]	43	F	15 yr	Heller's myotomy	No
McIntyre *et al*[10]	48	F	8 yr	Medication	Yes (7 yr)
Hooper *et al*[11]	45	F	19 yr	Heller's myotomy	Yes (15 mo)

[1]Achalasia was diagnosed after esophageal intramural hematoma (EIH) attack.

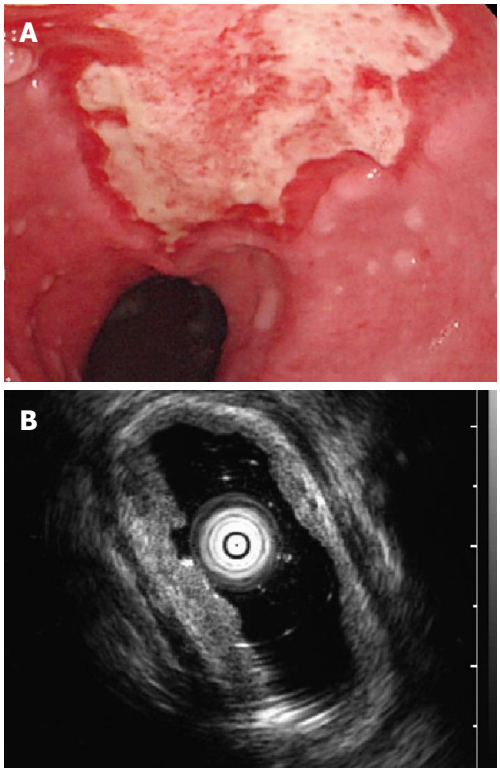

Figure 4 Esophagogastroduodenoscopy revealing a shallow ulcer without a tumor (A) and endoscopic ultrasound revealing thickening mucosal and submucosal layers, an intact muscularis propria layer, and no submucosal tumor (B) during the follow-up one week later.

and most of the patients are in the seventh or eighth decade of life. It has been reported that EIH is associated with coagulopathy[3], variceal injection sclerotherapy[4], and endoscopic instrumentation or foreign body ingestion[5]. It has also been known to occur spontaneously in healthy people[6]. EIH usually presents as a sudden onset of chest pain followed by odynophagia, dysphagia, and mild hematemesis.

EUS examination of EIH is limited. In our case, initial EUS showed a mixed echoic tumor lesion with an anechoic component and a mild irregular surface involving the mucosal and submucosal layers, but the muscularis propria was preserved and partial organization of hematoma was preferred rather than solid tumor. This patient also underwent follow-up EUS 1 wk later, which showed thickening of the mucosal and submucosal layers with a mucosal defect compatible with ulcer and complete resolution of hematoma.

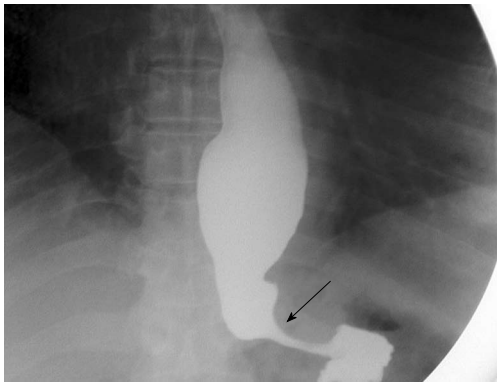

Figure 5 Esophagography revealing a dilated esophageal lumen with smooth narrowing of the esophagocardiac junction (arrow).

EIH occurs as a result of 2 types of trauma to the esophageal wall: barotrauma and direct trauma[7]. Esophageal barotrauma occurs due to a sudden increased transmural pressure associated with coughing, or retching. Direct trauma results from endoscopic injection sclerotherapy, or swallowing of pills or a large food bolus. Our patient was not known to have achalasia prior to this admission, possibly because he was symptom free due to long-term intake of calcium channel antagonists for controlling hypertension. He had intermittent chest pain and dysphagia for 1 mo. These symptoms may have been related to the underlying achalasia, but he denied having nausea, vomiting, or food regurgitation. However, he did experience a feeling of something being stuck in his esophagus. Furthermore, his platelet count and prothrombin time were normal, which prompted us to consider food bolus-related direct trauma to esophageal wall. To our knowledge, achalasia has been reported only in 4 female patients with EIH since 1957s[8-11] and three of them were already diagnosed with achalasia before the attack of EIH. Recurrent EIH has only been reported in 2 patients, with underlying achalasia[10,11] (Table 1). Fortunately, our patient fully recovered from EIH and underwent successful endoscopic dilatation for achalasia. He was in good condition, able to feed orally, and had no more chest pain or dysphagia at the time when we wrote this paper. However, long-term follow-up is needed.

The prognosis of patients with EIH is good, death has only been reported following thoracotomy for this condition[12]. Management should be conservative. Most patients can be treated using intravenous nutrition, analgesics with oral feeding discontinued. Bleeding from EIH is usually mild and spontaneously subsided, but

sometimes if bleeding is severe, endoscopic hemostasis or angiography with embolization even surgical intervention is necessary.

In conclusion, EIH is a rare disease that has different predisposing factors. EGD is a safe procedure for initial diagnosis. EUS findings help differentiate intramural hematoma from a solid tumor, cyst, or vessel. A series of follow-up endoscopies can help understand the natural course of EIH. Management is conservative, and the outcome is excellent with almost full recovery. However, it should be kept in mind that recurrence has been reported especially in patients with underlying achalasia.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

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Prague Hepatology Meeting 2010

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Prague, Czech Republic
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The 7th Biannual International Symposium of Society of Coloproctology

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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]

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

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

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EDITORIAL

Role of bacteria in carcinogenesis, with special reference to carcinoma of the gallbladder

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Abstract

Carcinoma of the gallbladder (CaGB) is the fifth commonest gastrointestinal tract cancer and is endemic in several countries. The interplay of genetic susceptibility, infections, and life style factors has been proposed to be responsible for carcinogenesis of gallbladder. Persistence of infection leading to chronic inflammation, and production of certain toxins and metabolites with carcinogenic potentials, by certain bacteria has been speculated to be involved in the transformation of the gallbladder epithelium. Therefore, any bacteria that have evolved to acquire both of the above carcinogenic mechanisms can cause cancer. *Salmonella typhi* has been found to be prominently associated with CaGB. Chronic typhoid carriage (persistence) and production of mediators of chronic inflammation and a genotoxic toxin (cytotoxic distending toxin, CdtB) are also known for this bacterium. Furthermore, the natural concentrating function of the gallbladder might amplify the carcinogenic effect of the mediators of carcinogenesis. In addition to *S. typhi*, certain species of *Helicobacter* (*H. bilis* and *H. hepaticus*) and *Escherichia coli* have also been implicated in carcinogenesis. As the isolation rate is very poor with the presently available culture techniques, the existence of bacteria in a viable but non-cultivable state is quite likely; therefore, sensitive and specific molecular techniques might reveal the etiological role of bacterial infection in gallbladder carcinogenesis. If bacteria are found to be causing cancers, then eradication of such infections might help in reducing the incidence of some cancers.

Key words: Bacteria; Chronic inflammation; Carcinogen; Bacterial toxins; Carcinoma gallbladder; DNA damage

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INTRODUCTION

Carcinoma of the gallbladder (CaGB) is the fifth commonest cancer of the gastrointestinal tract and it is one of the commonest biliary tract (BT) malignancies[1]. Although, gallbladder cancer was described as early as 1777[2], for the majority of patients late diagnosis and lack of effective treatment is a typical feature of the disease even today[2-4]. Carcinoma of the gallbladder is an aggressive disease with dismal prognosis and has marked ethnic and geographical variations in incidence. CaGB is more common in females than males[5], except in Far East Asian countries like Japan and China. The highest documented incidence rate was shown in women from

Delhi, India (21.5/100 000); followed by Karachi, Pakistan (13.8/100 000), and Quito, Equador (12.9/100 000)[5]. High incidence rates have also been reported from Far East Asia, Eastern Europe, South America, and Spain (Granada), the incidence of CaGB has also been reported to be high. However, the incidence rate of CaGB in North Europe and North America has been observed to be quite low (< 3/100 000)[6].

A number of factors, such as genetics, infections, and life style have been reported to be associated with CaGB. Genetic etiology might be more important in Japan, Korea, and China where the sex ratio for CaGB is close to unity. This cancer has also been linked with certain genetic disorders, such as multiple familial polyposis/Gardener syndrome[7], Peutz-Jegher syndrome[8], porcelain gallbladder[9], and anomalous pancreatico- biliary ductal union[10]. The interplay of genetic susceptibility, infections, and life style factors in gallbladder carcinogenesis is still poorly understood[6]. Despite recent insights into the possible mechanisms involved in biliary carcinogenesis, the key events and specific links in this multistage cascade that leads to transformation of gallbladder epithelial cells remain unknown and deserve further investigation. In this review, we have specifically focused on the association of chronic bacterial infection with CaGB.

CHRONIC MICROBIAL INFECTIONS AND CARCINOGENESIS

When bacteria were discovered to be the cause of many infectious diseases, it was accepted that cancer does not behave as an infectious or contagious disease. Thus, the notion of involvement of bacteria in carcinogenesis was rejected. In 1890, Russel[11] for the first time on the possibility of bacteria-induced carcinogenesis. A few years later, Thomas Glover[12] in 1926 stated that specific bacteria could be isolated consistently from neoplastic tissues. In 1931, Hodgkin's disease was found to be associated with acid fast bacteria[13]. Later, in 1941, George Mazet[14] reported that both leukemia and Hodgkin's diseases were consistently associated with bacteria. From 1936-1955, Crofton[15], Livingston *et al*[16], and Villesquez[17] also reported the presence of microbes in cancer tissues. In 1953, White[18] made a claim that antiserum raised against cancer bacteria had a protective effect. Diller[19] in 1953 reported the isolation of extremely polymorphic bacteria from cancer tissues.

However, in 1963, a group of scientists from National Cancer Institute (NCI), USA, rejected the hypothesis of association of bacteria with carcinogenesis. These bacteria were considered simply either as contaminants or having secondarily infected the cancer growth. In addition, this hypothesis could not withstand Koch's postulate. In contrast, a few years later (1969), another group of scientists from NCI, USA, reported positive associations between bacteria and cancers. Barile *et al*[20] in 1965 observed latent infection of Mycoplasma in leukemia cases. Later, Lo[21] in 1992 reported multistage malignant transformations due to Mycoplasma infection, which could be reversed by antibiotic therapy. The best studied relationship between bacterial infection and cancer is that of *Helicobacter pylori* implicated in two different forms of gastric cancers: MALT lymphoma and gastric adenocarcinoma[22]. *Streptococcus bovis* has been implicated in colon cancer, *Chlamydiae pneumonie* in lung cancer and *Bartonella species* in vascular tumor formation[22-25].

Certain animal studies have shown the involvement of *Helicobacter hepaticus* in chronic active hepatitis that progressed to hepatocellular carcinoma in A/JCr mice[26]. Chronic infection with *Citrobacter rodentium*, a mouse pathogen, which is genetically similar to enteropathogenic *Escherichia coli*, can result in colon cancer[27]. Recently, *H. hepaticus* has been stated to promote cancer formation indirectly in the mammary gland of mice[28].

For many years, chronic inflammation has been reported to be associated with a variety of epithelial malignant tumors. Chronic osteomyelitis has been found to be associated with the development of squamous cell carcinoma along the draining sinus of osteomyelitis[29], and chronic inflammatory bowel disease has been associated with increased risk of development of adenocarcinoma[30,31]. *Schistosoma haematobium,* a water-borne parasite that causes a secondary bacterial infection of the urinary tract due to its persistence, is an important cause of squamous cell carcinoma of the urinary tract in the Middle East and Northern Africa[32]. Furthermore, increase risk of developing cancer of the urinary bladder due to chronic inflammation has been confirmed by epidemiological data and by animal experiments[33-35].

MECHANISMS OF BACTERIAL CARCINOGENESIS

Understanding bacteria-induced carcinogenesis might enable us to prevent and cure some forms of cancers[36]. The involvement of bacteria in carcinogenesis is still not without controversy because no clear agreement has been achieved on the molecular mechanism/s by which they might promote carcinogenesis. In the 21st century, scientists started hypothesizing that: (1) Chronic inflammation caused by persistent bacterial infections might lead to carcinogenesis[37-39]; and (2) Bacterial toxins and secondary metabolites produced by the chronic bacterial infection might induce carcinogenesis[37].

Chronic inflammation due to bacteria and carcinogenesis

Bacterial infections are usually believed to cause acute disease, but it has now been accepted that many bacteria can cause chronic infections and diseases, including cancers[37,40].

There may be various mechanisms of carcinogenesis induced by chronic bacterial infections (Table 1[41-50] and Figure 1). Continuous release of mediators of inflammation is a common feature of chronic infections[37,38]. The nuclear factor-κB (NF-κB) family of transcription factors are linked to inflammation driven carcinogenesis[38]. The NF-κB activation pathway is triggered by microbial infections and also by proinflammatory cytokines, such as TNF-α and IL-1. This pathway leads to activation of IKK

Table 1 Chronic inflammatory mechanisms involved in carcinogenesis

Signaling	Sub categories	Role in inflammation assumed cancer
Pro-inflammatory cytokines and immunosuppressant cytokines	ILS: Pro-inflammatory (IL-1, IL-6, IL-8, IL-17); immunosuppressor (IL-10); TNF-α plays dual role in carcinogenesis, usually it is tumor promoter	Over expressed in inflamed and hyperplastic, metaplastic tissues and adenocarcinoma; Induce DNA damage; Pro-angiogenic molecule such as VEGF, VEGFR, IL-8, NO, ICAM-1 VCAM-1; Activation of pro-inflammatory signals mediated *via* JAK-STAT and NF-κB; Maintain inflammatory tumor microenvironment; Stimulate cell proliferation and inhibit apoptosis
Chemokines	Four major groups: CXC, CC, XC, CX3C (primary function is to recruit leucocytes at the site of inflammation)	Responsible for attraction to inflammatory and immune cells to tumor microenvironment; Promotion of tumor cell migration, facilitation of invasion and metastasis; Stimulation of inflammatory angiogenesis
COX-2 and prostaglandins	An inducible form of cyclooxygenase, serves as interface between inflammation and cancer[41-44]	Causes promotion of: cellular proliferation, suppression of apoptosis, enhancement of invasiveness, angiogenesis
iNOS	Expression of iNOS is elevated in various precancerous lesions and carcinomas[45]	Elevated in precancerous and cancerous lesions and cause: DNA damage by nitrosation/oxidative pathways; Produce proinflammatory mediators like NO by catalyzing Arginin metabolism; Acts as a downstream effector of NF-κB and inflammatory cytokines mediated signaling
NO	Elevated in precancerous and cancerous lesions[46]	Selects mutant p53 cells and contribute to tumorigenesis by upregulating VEGF; DNA damaged by nitrosation of nucleotide bases
NF-κB (The NF-κB/Rel family of proteins includes CRel, RelA (p65), RelB, NF-κB1 (p50/100), NF-κB2 (p52/p100)[47]	One of the DNA binding proteins that are aberrantly activated in response to inflammatory stimuli leading to induction of transcription of various proinflammatory genes in tumor cells[48]	Enhances expression/production of proinflammatory mediators: Amplifies inflammation signal transduction; Increased expression of anti-apoptotic protein; Help transformed cells to escape apoptosis
ErbB2 (a receptor strongly involved in carcinogenesis)	Inflammation induces the expression[49-50]	Binding of ErbB1 and ErbB2 to ligands results in prolong activation of intrinsic protein kinase activity, leading to activation of a biochemical cascade responsible for mitogenic cell signal transduction

ILS: Interleukins; IL: Interleukin; TNF: Tumor necrosis factor; CC: Chemotactic cytokine; NF-κB: Nuclear factor-κB; VEGFR: Vascular endothelial growth factor receptor; iNOS: Inducible nitric oxide synthetase; NO: Nitric oxide; VCAM-1: Vascular cell adhesion molecule 1; ICAM-1: Inter-cellular adhesion molecule 1.

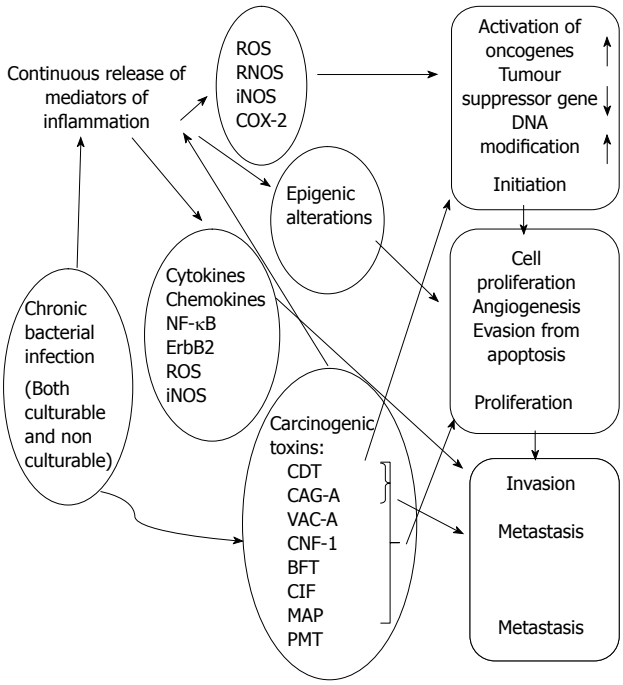

Figure 1 Mediators of inflammation and bacterial toxins in carcinogenesis caused by bacteria. ROS: Reactive oxygen species; RNOS: Reactive nitrogen oxide species; iNOS: Inducible nitric oxide synthetase; NF-κB: Nuclear factor-κB; CDT: Cytolethal distending toxin; CNF: Cytotoxic necrotizing factor; BFT: Bacteroides fragilis toxin; CIF: Cycle inhibiting factor; MAP: Mitochondrial associated protein; PMT: Pasturella multocida toxin.

complex[51] and to degradation of NF-κB inhibitors, thus freeing NF-κB to enter the nucleus and mediate transcription of target genes. Many of genes, such as cyclin D1, CDK2 kinase, c-myc (cell cycle regulators), involved in cell cycle control are upregulated, while the genes responsible for decreased apoptosis, such as p21, p53 and pRb, are downregulated by NF-κB. NF-κB also upregulates numerous cytokines, such as IL-1β, IL6, VEGF (proinflammatory and proangiogenic), but downregulates TNF, thus enhancing tumor growth. Genes responsible for invasion and metastasis are also upregulated by NF-κB. Moreover, downregulation of genes involved in suppression of apoptosis, i.e. Bcl-2 family members and IAP proteins, an important feature of cancer cells, is mostly due to deregulation by NF-κB. NF-κB not only helps in persistence of intracellular as well as extracellular infections, but also leads to suppression of cell death; thus creating a niche for bacterial survival defying the host immune response[52,53]. Survival of such partially transformed cells provides a chance for a higher level of transformation. Reactive oxygen species (ROS) and nitric oxide (NO) are produced by epithelial cells in response to inflammation. These compounds increase mutations in genes responsible for controlling malignant transformations. In particular, ROS can inhibit tyrosine phosphatases, causing overexpression of Mox1 (the catalytic subunit of NADPH oxidases). NO inhibits the Fpg protein, a DNA repair enzyme[54], leading to failure of damage control. HER-2/neu (also known

Table 2 Bacterial toxins and their possible roles in carcinogenesis

Toxin	Source	Activity and outcome
Potential genotoxins		
CDT (three subunits: CdtB is a functional unit, while CdtA and CdtC serve as accessory units for delivery into target cells)	*Haemophilus ducreyi, Helicobacter hepaticus, Salmonella typhi. Actinobacillus actinomycetemcomitans*	DNAase; DNA damage and cell cycle inhibitor[56,57]
Cytolethal distending toxin B	*Salmonella typhi*	DNAase activity, genotoxic by creating DNA lesions[58]
Colibactin	*Escherichia coli*	Mechanism unknown[59]
Potential pro-carcinogenic toxins		
Pasturella multocida toxin	*Pasturella multocida*	Modifies Gq proliferation[60]
CagA	*Helicobacter pylori*	Binds to SHP2 and c- Met cells scattering[61]
Vacuolating cytotoxin A	*Helicobacter pylori*	Upregulation of VEGF expression (seems to depend on the activation of EGFR, MAP kinase and COX-2 mediated)
Bacteroides fragilis toxin	*Bacteroides fragilis*	Cleaves E- cadherin proliferation[62]
Cytotoxic necrotizing factor-1	*Escherichia coli, Shigella dysenteriae, Campylobacter jejuni and Salmonella typhi, Helicobacter hepaticus, Actinobacillus actinomycetemcomitans*	Modifies Rho family proteins, inflammation and inhibition of cell cycle, blocks cytokines[39]
Cycle inhibiting factor	*Escherichia coli*	Inhibit cell cycle at G_2-M transition[63]
MAP	*Citrobacter rodentium*	Multifunctional effectors protein that target host cell mitochondria implicated in the disruption of epithelial barrier function both *in vitro* and *in vivo*[64]
VEGF	*Bartonella species*	Angiogenesis and proliferation[65]

MAP: Mitochondrial associated protein; VEGF: Vascular endothelial growth factor; EGFR: Epidermal growth factor receptor.

as ErbB2) stands for "human epidermal growth factor receptor 2" and is a protein conferring higher aggressiveness in breast cancers. It is a member of the ErbB protein family, more commonly known as the epidermal growth factor receptor family, which is involved in cell proliferation, differentiation, and oncogenesis. Overexpression of ErbB2 can occur due to chronic inflammation. Binding of ErbB1 and ErbB2 to ligands results in prolonged activation of intrinsic protein kinase activity, leading to activation of a biochemical cascade responsible for mitogenic cell signal transduction[49,50]. All these factors contribute to the multistage process of carcinogenesis[55]. These factors all cause oxidative damage to DNA of the cells (Figure 1).

Bacterial toxins implicated in carcinogenesis

The probable bacterial toxins implicated in carcinogenesis are listed in Table 2[39,56-65]. The pathways involved in carcinogenesis have been depicted in Figure 1. Bacterial toxins can either kill the cells or modify the cellular processes that control DNA damage, proliferation, apoptosis, and differentiation. These toxins interfere either with the key eukaryotic processes, such as cellular signaling components, or directly by attacking the DNA[62,66]. The damage to the host cells can be mediated either[67]: (1) directly by: (a) enzymatic attack; (b) DNA damage; or (c) by affecting DNA damage repair mechanisms; or (2) indirectly by: (a) provoking a chronic inflammatory reaction; or (b) producing free radicals.

These changes might be associated with carcinogenesis and might stimulate cellular aberrations, modify the immune response, or inhibit normal cell controls.

BACTERIOLOGY OF THE GALLBLADDER AND BILE

Lykkegaard *et al*[68] reported that the liver is normally sterile, as is bile from individuals with a normal biliary tree. Bile favors the growth of some organisms but inhibits others, such as *Streptococcus pyogenes* and *Streptococcus pneumoniae*.

A positive bile culture might not only be important in the genesis of biliary tree infection, but might also be a significant determining factor in the incidence of various short and long term consequences. The pathological process of cholecystitis can be acute, chronic, or more commonly, a combination of both types. This is evident from the observations that approximately 95% of gallbladders removed for acute cholecystitis exhibit fibrosis and other signs of chronic inflammation.

Routes of infection through which microbes may enter the biliary tract

(1) Ascending route - although the sphincter of Oddi, situated at the junction of the biliary tract and the gastrointestinal tract, forms an effective mechanical barrier to duodenal reflex vis-à-vis ascending bacterial infection, when the barrier mechanism is broken down either by surgical intervention or by certain pathology, microbes can enter the biliary system. It is interesting to note that the type of organisms recovered from bile are not those dominant in the sparse flora of the duodenum but are usually encountered in the ileum and colon[69] (Table 3); and (2) Descending route (hematogenous route) - As a part of the normal innate immune system, Kupffer cells prevent toxic metabolites and bacteria from entering the hepatobiliary system from the portal circulation. Added to this, the continuous flushing action of bile and the bacteriostatic effect of bile salts keep the biliary tract sterile under normal conditions. Moreover, secretory immunoglobulin A (SIgA), the predominant immunoglobulin in the bile and the mucous membrane and excreted by the biliary epithelium, probably acts by its anti-adherent function to prevent microbial

Table 3 Spectrum of bacteria isolated from bile (Brook *et al*[73])

Organism	No. of isolates	%
Aerobic bacteria		
Escherichia coli	71	32.9
Group D streptococci	42	19.4
Klebsiella species	29	15.3
Enterobacter species	26	12.5
Proteus species	15	6.9
α-haemolytic streptococci	11	5.1
Citrobacter species	8	3.6
Staphylococcus species	7	3.2
γ-haemolytic streptococci	5	2.3
Pseudomonas species	2	0.9
Anaerobic bacteria		
Clostridium perfringens	23	29.9
Bacteroides fragilis	9	11.7
Other *Bacteroides* species	5	6.5
B. thetaiotamicron	4	5.2
B. ovatus	2	5.2
B. distasonis	2	2.6
Propionibacterium acne	7	9.1

colonization. Despite these mechanisms, it is likely that organisms in the bile might be derived from blood.

The presence of bacteria in bile may not cause symptoms. In a series of cases, Flemming *et al*[70] observed that only 20 of 32 patients with positive cultures had symptomatic cholangitis, while six of 43 with negative cultures had had symptoms in the recent past. Further, out of 15 patients who had previous biliary intestinal anastomosis, 12 had positive cultures, but only seven had a history of cholangitis. However, efficiency of culture isolation techniques and the type of bacteria associated specifically with symptoms must be explored further. Gallstone formation has been reported to be predisposed by bactobilia[71]. Bacteria themselves might act as a nidus for gall stone formation or may alter the bile composition or damage the wall of the gallbladder. It is difficult to estimate the bacteriology of bile in an absolutely healthy population. The majority of the available reports regarding the microbial spectrum of infected bile are from individuals suffering from hepatobiliary diseases. Interestingly, most of these reports concur with one another[72].

Brook[73] reported the spectrum of anaerobic isolates from the biliary tract (Table 3). Anaerobic bacteria could be recovered from 48% of specimens. Anaerobic bacteria could be isolated exclusively in 3% and mixed in 49% of 123 bile specimens collected. The author suggested that lowering of oxygen tension and pH achieved by initial colonization of aerobic bacteria in acute infection paved the way for predominance of anaerobes in the chronic stage of illness. However, it is difficult to draw any conclusion without knowing the flora of gallbladder in individuals without any sign or symptom. Most studies have reported isolation of the bacteria in bile to be < 50%[74,75]. Lu *et al*[76] detected bacterial DNA in 78.3% of CaGB tissue samples. They used a single amplification cycle targeting 16S r DNA. However, detection rates will rise further if nested PCR (being more sensitive) is used. Therefore, the possibility of the existence of viable but non-culturable (VBNC) forms of bacteria cannot be ruled out in the gallbladder. However, the detection of bacteria causing persistent infection in biliary system is warranted.

Gallbladder cancer and Salmonella typhi

The interplay of genetic susceptibility, life style factors, and infections of the hepatobiliary system in carcinogenesis of the gallbladder is poorly understood; however, a link has been specifically proposed between chronic bacterial infections of the biliary tree and *S. typhi*. An association of chronic typhoid carriage and carcinoma of the gallbladder was first reported by Axelrod *et al*[77]. Welton *et al*[78] observed increased incidence of cancer of the hepatobiliary system in typhoid carriers; this was later confirmed by other studies[79,80]. Caygill *et al*[81] studied cancer mortality in people infected during the Aberdeen typhoid outbreak in 1964; their results suggested a lifetime risk of developing gallbladder cancer in 6% of the carriers. Strom *et al*[82] from Bolivia and Mexico have reported a 12-fold increase in CaGB in subjects with a history of typhoid fever. However, they could not prove the same by serology. Moreover, Shukla *et al*[83] from Northern India, using Vi serology, showed a 7.9 times increased risk for CaGB in chronic typhoid carriers. Earlier also from North India, Nath *et al*[84] demonstrated significantly higher isolation rates of *Salmonella typhi* and *paratyphi*-A from bile, gallbladder tissue, and stones from patients with CaGB as compared to those suffering from benign gallbladder diseases. The relative risk of developing CaGB was reported to be 9.2. Based on serology, Dutta *et al*[85], from North India, reported a 14-fold increased risk of CaGB in a case-control study. In Japan, an area with an extremely low prevalence of typhoid fever, in a large cohort of 113 394, the relative risk of developing CaGB was reported to be 2.1[86]. Recently, Nath *et al*[87] reported the prevalence of chronic typhoid carriers in CaGB patients using a very sensitive and specific nested PCR technique, in hepatobiliary specimens, to exclude the limitations of serology based detection and culture isolation (low sensitivity of culture and variable individual immune response, depending on the stage of the disease). They showed that 67.3% of the CaGB patients were typhoid carriers, as compared to 8.3% of the healthy population (hepatobiliary specimens from dead bodies; victims of unnatural deaths) in the typhoid endemic area of North India (Odds ratio 22.8). In the same study, the authors tried to locate the niche of the *S. typhi* bacterium in chronic typhoid carriers, and found that the bacterium was most prevalent in the liver[88]. Therefore, it could be proposed that *S. typhi* lives in the liver and is excreted into the gallbladder intermittently. Metabolites (mutagens and inflammation inducers) and toxins produced by the multiplying bacteria are further concentrated about 10 times in the gallbladder, which thereby bears the major brunt of the mutational changes. Various carcinogens produced by *S. typhi* have been suggested: Bacterial glucuronidase, yielding some high energy intermediates after acting on bile[89],

bacterial enzymes acting upon primary bile acids and producing carcinogenic secondary bile acids at very high concentrations[90], and the production of nitroso compounds from nitrates by the action of bacterial enzymes[91]. Chronic bacterial infection leads to obstruction and persistent chemical and mechanical injuries[92].

Cytolethal distending toxin (CDT), the first bacterial genotoxin described, is also produced by *S. typhi*[58] in addition to *Escherichia coli*[93] and other causative agents of chronic infection, such as *Campylobacter jejuni*[94], *Haemophilus ducreyi*[95], *Shigella dysenteriae*[96], *Actinobacillus actinomycetemcomitans*[97], *Helicobacter hepaticus*[98], and other species[99,100]. The holotoxin is a tripartite complex, where the CdtB subunit, a structural and functional homolog of mammalian DNAse I, is the active subunit, while CdtA and CdtC mediate the binding of the holotoxin to the plasma membrane of the target cells. In a cell culture study (Cos2 and Henle-407 cell lines), Haghjoo *et al*[58] found that *S. typhi* produced a unique CdtB-dependent CDT that required bacterial internalization into host cells. When Cos-2 cells were transfected with *S. typhi*, the effects of the CdtB subunit were severe fragmentation of chromatin, a typical characteristic of the CdtB subunit of CDT expressed by other species. The authors proposed that *S. typhi* subsequent to internalization deviated from the usual endocytic pathway that leads to lysosomes, reaching an unusual membrane-bound compartment where it can survive and replicate due to its ability to produce abundant antiphagocytic Vi capsule. What is the role of CDT in *S. typhi* pathogenesis? It is worth mentioning that *S. typhi* is the only serovar of Salmonella that encodes CdtB. Furthermore, *S. typhi* is a human-restricted pathogen, causing chronic persistent infections. CDT might facilitate the persistence of infection, because this toxin is known for its immunomodulatory activity[97]. CdtB, after being delivered to the cytosol, reaches the nucleus of the target cell where it causes DNA damage[101]. Therefore, in typhoid endemic areas, *S. typhi* might be one of the important etiologic factors for CaGB.

Gallbladder cancer and Helicobacter species

Helicobacter pylori (*H. pylori*) infection is a well-established cause of stomach cancer[102]. Since the discovery of *H. pylori* in 1982, thirty other *Helicobacter* species have been identified from the stomach, intestinal tract, and liver of mammals and birds. A few species found in human bile and biliary tract tissue biopsies (*H. bilis, H. pullorum, H. hepaticus, H. pylori etc.*) have been suspected to cause biliary tract diseases. As discussed earlier, any bacteria, *Helicobacter* spp. in particular, causing persistent infection in the biliary tract might induce chronic inflammation and gallstone formation, especially due to urease production[103,104]. Gallstones further aggravate chronic inflammation and can induce transformation, which is further amplified many fold by several toxins and metabolites of known carcinogenic potentials produced by the Helicobacters[105,106]. *H. hepaticus* is a known agent causing chronic active infection of biliary canaliculi progressing to liver cancer[2]. PCR-based detection rates of different species of *Helicobacter* spp. in biliary tract cancer vary from 0%-82.8%[107]. Using species-specific primers, *H. bilis* was found in 35 out of 67 specimens (52.2%) from four different studies, whereas *H. hepaticus* was searched for in two studies, but only in one study were four out of 19 specimens (21.1%) found to be positive for the bacterium. In contrast, Pradhan *et al*[108] from Nepal have shown *Helicobacter hepaticus* infection in 82% of non-malignant gallbladders and in 87.5% of malignant cases. Whether *Helicobacter hepaticus* is the number one cause of the type of gallstone formation that ultimately leads to malignancy, or is itself a risk factor for the pathogenesis of carcinoma gallbladder, is yet to be determined. Murata *et al*[109] showed that *H. bilis* specific sequences could be amplified in three of 11 (27.2%) gallbladder cancer cases and in one of three (33.3%) cases with biliary duct cancer. One study conducted in Japanese and Thai populations showed that patients positive for *H. bilis* had a 6-fold higher risk of biliary tract carcinoma. However, it is premature to make conclusion about the role of *Helicobacter species* in causing CaGB. *H. pylori* infection was also identified as a risk factor for biliary tract cancer and the corresponding relative risk (RR) was 9.9 (95% CI: 1.4-70.5) after adjustment for age and sex. *H. bilis* and *H. pylori* have been identified in bile specimens and associated with risk of biliary tract cancer. Another study[109] found a positive association between *H. bilis* and CaGB, with a crude RR of 2.6 (95% CI: 0.6-4.6). Larger epidemiological studies are required before Helicobacters can be in gallbladder cancer, but only after the development and validation of specific serological tests and direct detection of these bacterial species in the gallbladder itself.

Gallbladder cancer and Escherichia coli

E. coli is the normal inhabitant of the human intestine and can become highly pathogenic following the acquisition of virulence factors, usually by horizontal gene transfer. Cytotoxic necrotizing factor 1 is one of the important protein toxins acquired in this way. *Escherichia coli* is the commonest species isolated from gallbladder specimens and CDT is present in many isolates of *E. coli*. Lax[37] reported a novel genotoxin, named as colibactin. The mechanism of action of this toxin is yet to be explored; however, it causes double stranded DNA breaks. It is likely that some of these acquired gene(s) enable these strains cause persistent infection, and facultative or obligate intracellular invasion, leading in turn to more chances of transformation of the host cells. In support of above speculation, Yamamoto *et al*[110] have shown that there was marked enhancement of rat urinary bladder carcinogenesis by heat killed *E. coli*. Furthermore, the occurrence of chronic urinary tract infections leading to carcinogenesis lends support to the above suggestion[111].

Gallbladder cancer and other bacteria

Several bacteria and their products display potentially carcinogenic characteristics. There is ample evidence to support the view that some bacteria can establish chronic infection, often without overt sign of the disease. In fact,

many bacteria form the chronic carrier state, usually in viable but non-cultivable (VBNC) states or in a cell wall deficient form. They are relatively dormant but retain their virulence[112,113]. The poor yield of bacterial isolation as compared to detection by PCR affirms this possibility[87]. Therefore, any bacteria (aerobic or anaerobic) acquiring genes that enable them to cause persistent infection, and are capable of producing carcinogenic toxin, secondary metabolites, and most importantly chronically released inflammatory mediators, might be able to transform the host cells. It must be stressed that the isolates from affected sites with chronic infection or cancer must be characterized in terms of their ability to colonize, and for the production of metabolites with carcinogenic potential.

CONCLUSION

Carcinoma of the gallbladder is one of the commonest malignancies of the biliary tract. The main associated risk factors identified to date include chronic cholelithiasis, chronic infection, obesity, hormonal factors, environmental exposure to specific mutagens, and genetic predisposition. Tumorigenesis is a long and complex process, and the gap between initiation and development of cancer might hide the role of microbial infection. Therefore, a direct link between bacterial infection and cancer is often not detectable, and the etiological role of the former in causation of cancer is mostly underestimated. However, chronic infections lead to the persistent release of mediators of inflammation, toxins, and metabolites and these factors may be potentially mutagenic and/or cell cycle modulators. A strong association between chronic Salmonella carriage and cancer of the gallbladder has now been proposed. However, it is likely that other bacteria in addition to *S. typhi*, which persistently inhabit the gallbladder, might be important etiological factors. Attempts have been made to conquer cancer over many decades, but the conventional strategies like chemotherapy and radiotherapy, often cannot prevent or cure cancer. Eradication of causative microbes by antibiotic therapy, and immunological potentiation by active, as well as passive, methods, will definitely lead to reduction in the incidence of bacteria-induced cancers. In addition to this approach, numerous anti-inflammatory agents of natural and synthetic origin are reported to have inhibitory effects on inflammation-induced carcinogenesis. Cellular miRNAs might also have the potential to control and prevent carcinogenesis. Knowing the genetic susceptibility for persistence of a specific bacterial agent will help in the choice of prophylactic measures in such individuals. Thus, limiting the reservoir and transmission of such potentially pathogenic microorganisms will help in decreasing the incidence of chronic and acute diseases.

FUTURE PERSPECTIVE

There is a strong need for in-depth studies looking into the role of persistent bacterial infections and carcinogenesis of the same or related parts of the body. Further study is required into the mechanism of chronic inflammatory mediators and bacterial toxins in cell transformations. Detailed study needs to be carried out to delineate whether it is the infection or the disease that occurs first in the case of gallbladder pathology. Effort should be made to substantiate the exact role of bacteria like *Salmonella typhi*, *Escherichia coli*, and *Helicobacter* species in the causation of biliary tract cancer in suitable animal models. Once the role of chronic bacterial infection in carcinogenesis is established, ways could be found to cure or eradication such agents from the community by chemotherapy, immunotherapy, and hygienic practices. Anti-inflammatory therapeutic approaches to cancer development, which can block/modify carcinogenic mechanism/s, such as anti-inflammatory agents or miRNA, should be explored.

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EDITORIAL

Prevention of recurrence after surgery for Crohn's disease: Efficacy of infliximab

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Abstract

After surgery for Crohn's disease (CD), early endoscopic lesions are frequently observed despite no symptomatic recurrence. The severity of lesions found at postoperative endoscopy is reported to be a strong predictive factor for future clinical recurrence. If endoscopic lesions in the early postoperative period can be reduced with medications, symptomatic recurrence will likely be delayed and decreased. Before the introduction of biologic therapies, various medications were used for the maintenance of clinical remission after surgery; however, few demonstrated consistent efficacy. Infliximab is a recombinant anti-tumor necrosis factor-α antibody. Although infliximab is one of the most effective medications in the management of CD, its efficacy for early endoscopic lesions after surgery has not yet been assessed. The author and colleagues recently conducted a prospective study in order to investigate the impact of infliximab on early endoscopic lesions after resection for CD. We found that infliximab therapy showed clear suppressive effects on clinical and endoscopic disease activity in patients with early endoscopic lesions after resection.

Key words: Crohn's disease; Postoperative recurrence; Endoscopic lesion; Infliximab

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INTRODUCTION

Crohn's disease (CD) is a chronic relapsing, remitting inflammatory bowel disease, the cause of which remains unknown. Nearly 80% of patients with CD require surgery during their lifetime[1]. CD can be palliated but not cured by surgery because inflammation tends to return in areas adjacent to those that were previously removed. Postoperative recurrence is common, and many patients require repeat operations. Reoperation rates for recurrence are reported to be 10%-35% at 5 years, 20%-45% at 10 years and 45%-55% at 20 years[1].

Although postoperative recurrence is common in CD, the determinants of disease recurrence remain speculative. Several patients with CD experience frequent recurrences while others have prolonged periods of remission after surgery. Identifying risk factors for postoperative recurrence would be useful to identify patients at a high risk of progressive recurrence and to determine strategies for medical therapy after surgery.

RISK FACTORS FOR POSTOPERATIVE RECURRENCE

The author and colleagues have conducted systematic reviews and meta-analyses of clinical trials reporting on

surgical outcomes for CD in order to determine risk factors for postoperative recurrence[1-5].

Smoking

The most significant factor affecting postoperative recurrence of CD is smoking. In a meta-analysis[2], 16 studies encompassing 2962 patients including 1425 non-smokers (48.1%), 1393 smokers (47.0%) and 137 ex-smokers (4.6%) were investigated. Smokers had significantly higher clinical recurrence than non-smokers [odds ratio (OR): 2.15, 95% confidence interval (CI): 1.42-3.27]. Smokers were also more likely to experience surgical recurrence within 5 years (OR: 1.06, 95% CI: 0.32-3.53) and 10 years (OR: 2.56, 95% CI: 1.79-3.67) of follow-up compared to non-smokers. There was no significant difference between ex-smokers and non-smokers in the reoperation rate at 10 years or in the rate of postoperative acute relapses. In other clinical trials[6,7], quitting smoking reduced the recurrence rate in patients with CD.

Indication for surgery

In one meta-analysis[3], 13 studies reported on 3044 patients, 1337 (43.9%) of whom had perforating disease (perforation, fistula or abscess) and 1707 (56.1%) had non-perforating indications for surgery. The probability of reoperation for recurrence was found to be significantly higher in patients with perforating indications compared to those with non-perforating indications [hazard ratio (HR): 1.50, 95% CI: 1.16-1.93]. The indication for reoperation in CD tends to be the same as the primary operation, i.e. perforating disease tends to re-present as perforating disease, and non-perforating as non-perforating.

Granuloma in the specimen

In a recent meta-analysis[4], 21 studies reported 2236 patients with CD, of whom 1050 (47.0%) had granulomas (granulomatous group) and 1186 (53.0%) had no granulomas (non-granulomatous group). The number of recurrences and reoperations was found to be significantly higher in the granulomatous group compared to the non-granulomatous group (OR: 1.37, 95% CI: 1.02-1.84 and OR: 2.38, 95% CI: 1.43-3.95, respectively).

Duration of disease before surgery

A number of studies have shown a higher risk of postoperative recurrence when the duration of the disease before surgery was short[1]. There were, however, different definitions of "short" among the studies.

Anastomotic configuration

The author and colleagues conducted a meta-analysis to investigate the impact of anastomotic type on the incidence of perianastomotic recurrence[5]. Eight studies reporting on 661 patients who underwent 712 anastomoses, of which 383 (53.8%) were sutured end-to-end anastomosis and 329 (46.2%) were other anastomotic configurations were included. There was no significant difference between the anastomotic configurations in perianastomotic recurrence and reoperation for perianastomotic recurrence. Furthermore, a recent randomized controlled trial (RCT)[8] compared endoscopic and symptomatic recurrence rates between patients who had stapled side-to-side anastomosis and hand-sewn end-to-end anastomosis. The endoscopic recurrence rate was 42.5% in the end-to-end anastomosis group compared with 37.9% in the side-to-side anastomosis group (not significant). The symptomatic recurrence rate was 21.9% in the end-to-end anastomosis group compared with 22.7% in the side-to-side anastomosis group (not significant). Based on these results, the anastomotic technique following bowel resection does not seem to affect postoperative recurrence.

Other factors

The following factors do not seem to be predictive of postoperative recurrence: age at onset of disease, sex, family history of CD, anatomical site of disease, length of resected bowel, blood transfusions and postoperative complications[1].

In summary, risk factors for postoperative recurrence are smoking, perforating disease and granuloma in the resection specimen.

ENDOSCOPIC FINDINGS AFTER SURGERY

Rutgeerts *et al*[9] reported that recurrent lesions were observed endoscopically in the neo-terminal ileum (the proximal site of the ileocolonic anastomosis) within 1 year of resection in 73% of patients, although only 20% of the patients had symptoms. Three years after surgery, the endoscopic recurrence rate increased to 85% and symptomatic recurrence occurred in 34%. In their study[9], patients with severe endoscopic lesions within 1 year after resection developed early clinical recurrence. In contrast, patients with no or mild endoscopic lesions had a low frequency of subsequent clinical recurrence. The severity of the endoscopic inflammation in the neo-terminal ileum during the first year after resection was found to be a reliable predictive risk factor for future clinical recurrence.

The Rutgeerts score is a well-established endoscopic scoring system based on examination of the neo-terminal ileum; i0: no lesions; i1: < 5 aphthous lesions; i2: > 5 aphthous lesions with normal mucosa between lesions, or skip areas of larger lesions or lesions confined to < 1 cm from the ileocolonic anastomosis; i3: diffuse aphthous ileitis with diffusely inflamed mucosa; and i4: diffuse inflammation with larger ulcers, nodules, and/or narrowing[9]. This scoring system is widely used in clinical practice, and endoscopic recurrence after resection for CD is defined as a score of i2, i3, or i4.

The author and colleagues[10] conducted a prospective study to investigate the relationship between endoscopic findings in the neo-terminal ileum (the proximal site of the anastomosis) and subsequent clinical recurrence rates following ileocolonic resection for CD. Forty patients who had maintained clinical remission defined as CD activity index (CDAI)[11] < 150 with 3 g/d mesalamine during 6 mo

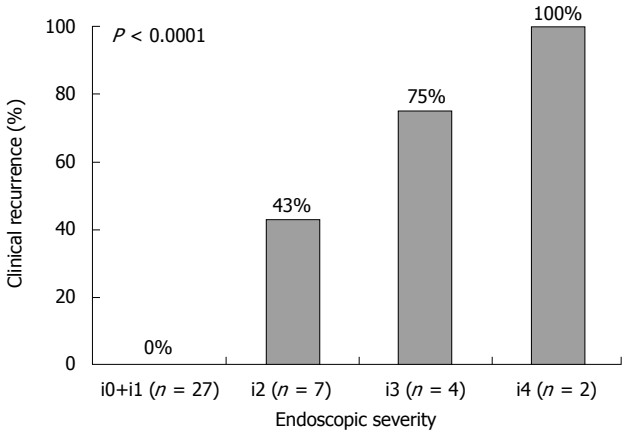

Figure 1 Correlation between the endoscopic severity in the neo-terminal ileum at 6 mo after ileocolonic resection and the clinical recurrence rate during the following 1 year.

after ileocolonic resection were studied. Six months after surgery, ileo colonoscopy was performed, and the endoscopic activity score in the neo-terminal ileum was determined according to the Rutgeerts score. All patients were regularly monitored, and clinical disease activity was assessed. Clinical recurrence was defined as CDAI ⩾ 150. Corticosteroids, immunosuppressants or tumor necrosis factor (TNF)-α blocking agents were not given unless there was clinical recurrence. Six months after surgery, the endoscopic scores were i0 or i1 in 27 patients, i2 in 7 patients, i3 in 4 patients, and i4 in 2 patients. There was a significant positive correlation between the endoscopic severity of the neo-terminal ileum 6 mo after surgery and the clinical recurrence rate during the following 1 year (Figure 1). From these results, the assessment of endoscopic lesions in the neo-terminal ileum appeared to be valuable for predicting subsequent clinical recurrence after ileocolonic resection for CD. Patients who postoperatively develop early endoscopic lesions despite mesalamine therapy do not benefit from continuing mesalamine. For such patients, more aggressive therapies such as TNF-α blocking agents should be considered. Thus, the early endoscopic inflammation in the neo-terminal ileum after ileocolonic resection is a suitable model to investigate the pathogenesis of CD, and also to evaluate new therapeutic modalities for prevention of progressive recurrence.

MEDICAL TREATMENT FOR PREVENTION OF POSTOPERATIVE RECURRENCE

Mesalamine, antibiotics, immunosuppressants, elemental diet

A Cochrane systematic review[12] was conducted to investigate the efficacy of medical therapies for the prevention of postoperative recurrence of CD. Twenty-three RCTs that compared medical therapy with placebo or other medical agents for the prevention of recurrence were included. Mesalamine therapy was associated with a significantly reduced risk of clinical recurrence [relative risk (RR): 0.76, 95% CI: 0.62-0.94, number needed to treat (NNT) = 12], and severe endoscopic recurrence (RR: 0.50, 95% CI: 0.29-0.84, NNT = 8) when compared with placebo. Nitroimidazole antibiotics appeared to reduce the risk of clinical recurrence (RR: 0.23, 95% CI: 0.09-0.57, NNT = 4) and endoscopic recurrence (RR: 0.44, 95% CI: 0.26-0.74, NNT = 4) when compared with placebo. However, these agents were associated with a higher risk of serious adverse events (RR: 2.39, 95% CI: 1.5-3.7). Azathioprine/6-mercaptopurine (6-MP) was also associated with a significantly reduced risk of clinical recurrence (RR: 0.59, 95% CI: 0.38-0.92, NNT = 7) and severe endoscopic recurrence (RR: 0.64, 95% CI: 0.44-0.92, NNT = 4) when compared with placebo. Neither agent had a higher risk than placebo of serious adverse events. When compared to azathioprine/6-MP, mesalamine was associated with a higher risk of any endoscopic recurrence (RR: 1.45, 95% CI: 1.03-2.06), but a lower risk of serious adverse events (RR: 0.51, 95% CI: 0.30-0.89).

The author and colleagues[13] conducted a prospective non-RCT to investigate the impact of enteral nutritional therapy with elemental diet on postoperative recurrence of CD. After resection, 20 patients continuously received enteral nutritional therapy (EN group), and 20 had neither nutritional therapy nor food restriction (non-EN group). In the EN group, an elemental diet was infused through a nasogastric tube in the night-time, and low fat foods were taken in the daytime. The clinical recurrence rate during 1-year follow-up was significantly lower in the EN group than in the non-EN group (5% *vs* 35%). One year after surgery, the endoscopic recurrence rate was also significantly lower in the EN group than in the non-EN group (30% *vs* 70%). We found that enteral nutrition significantly reduced postoperative clinical and endoscopic recurrences. However, an RCT with a larger number of patients is necessary to assess the definite efficacy of enteral nutritional therapy for the prevention of postoperative recurrence.

Infliximab

Infliximab is a recombinant anti-TNF-α antibody, and it reduces intestinal inflammation in patients with CD by binding to and neutralizing TNF-α on the cell membrane and in the blood, and by destroying TNF-α producing cells. Infliximab is indicated for treatment of moderately to severely active CD for the reduction of signs and symptoms in patients who have an inadequate response to conventional medications. In maintenance studies[14-16], a re-treatment regimen of infliximab can provide long-term suppression of disease activity in patients with CD. At the present time, infliximab is one of the most effective medications in the management of CD. However, the impact of infliximab on recurrence in the postoperative setting had not yet been reported.

Recently, Sorrentino *et al*[17] conducted a prospective pilot study, in which infliximab was administered after surgery along with low-dose methotrexate, while controls were treated with mesalamine alone. Infliximab was given as an intravenous infusion (5 mg/kg), with an intravenous

100 mg bolus of hydrocortisone starting from 2 wk after surgery, followed by standard maintenance treatment (2, 6, and then every 8 wk) and therapy with low-dose methotrexate (10 mg/wk, orally). Patients in the control group were given mesalamine-coated tablets, 800 mg 3 times daily, starting from 2 wk after surgery. In the group treated with infliximab and low-dose methotrexate, none of 7 patients had endoscopic or clinical recurrence during 2 years after surgery. In contrast, in the group treated with mesalamine, only 4 of the 16 patients (25%) were disease free during 2 years after surgery. Among 12 patients with recurrent disease, 7 had endoscopic recurrence, while 5 had both endoscopic and clinical recurrences.

Subsequently, Regueiro *et al*[18] conducted a randomized, double-blind, placebo-controlled trial. Twenty-four patients with CD who had undergone ileocolonic resection were allocated to receive intravenous infliximab (5 mg/kg, n = 11), administered within 4 wk of surgery and continued for 1 year, or placebo (n = 13). The primary endpoint was endoscopic recurrence (Rutgeerts score of i2, i3, or i4) at 1 year. Secondary end points were clinical recurrence (CDAI > 150) and histologic recurrence (scoring system according to D'Haens[19]). The endoscopic recurrence rate at 1 year was significantly lower in the infliximab group (9.1%) compared with the placebo group (84.6%). There was a non-significantly lower proportion of patients with clinical recurrence in the infliximab group (20.0%) compared with the placebo group (46.2%). The histologic recurrence rate at 1 year was significantly lower in the infliximab group (27.3%) compared with the placebo group (84.6%). From these results, 1-year infliximab treatment after surgery was effective for preventing endoscopic and histologic recurrences of CD. Albeit a small sample size, this study provides the strongest evidence for the efficacy of postoperative infliximab therapy.

EFFICACY OF INFLIXIMAB FOR EARLY ENDOSCOPIC LESIONS

Infliximab may be useful for the prevention of postoperative recurrence of CD[17,18]. However, this new agent cannot be recommended for all patients after surgery because of potential adverse events and high medical costs. A number of patients can maintain remission without medications or with mesalamine after surgery as shown in our study[10]. Infliximab should be used for patients at high risk of postoperative recurrence. Although smoking and perforating disease are risk factors for recurrence[1-3], these parameters are not so useful and are of limited use in clinical practice. A more practical predictor of postoperative recurrence is the severity of endoscopic inflammation in the early postoperative period[9,10].

Recently, the author and colleagues[20] conducted a prospective pilot study to investigate the efficacy of infliximab on early endoscopic recurrence after ileocolonic resection. Twenty-six patients maintaining clinical remission (CDAI < 150) with mesalamine (Pentasa® 3 g/d) after surgery showed endoscopic recurrence (Rutgeerts score of i2, i3, or i4) in the neo-terminal ileum at 6 mo postoperatively (study baseline). Patients were then allocated to one of 3 treatment groups. In Japan, azathioprine and infliximab maintenance therapy was approved by the Ministry of Health, Labor and Welfare quite recently (in June 2006, and November 2007, respectively). During the first study period (from January 2005 to June 2006), patients were continuously treated with mesalamine (Pentasa® 3 g/d) (mesalamine group, n = 10) for the following 6 mo. During the second period (from June 2006 to June 2007), azathioprine therapy (Imuran® 50 mg/d) was used (azathioprine group, n = 8), and during the last period (from June 2007 to May 2008), infliximab therapy (Remicade® 5 mg/kg, every 8 wk) was introduced (infliximab group, n = 8). In each treatment group, administration of azathioprine or infliximab was started at 6 mo after surgery, and then continued for 6 mo. At baseline and 6 mo later, endoscopic examination was conducted for all patients. During the 6-mo observation, no patients in the infliximab group, 3 (38%) in the azathioprine group, and 7 (70%) in the mesalamine group developed clinical recurrence (CDAI $\geqslant$ 150, P = 0.01). At 6 mo, endoscopic inflammation was improved in 75% of patients in the infliximab group, 38% in the azathioprine group, and 0% in the mesalamine group (P = 0.006). Complete mucosal healing was achieved in 38% of patients in the infliximab group, 13% in the azathioprine group, and 0% in the mesalazine group (P = 0.10). These results clearly showed that infliximab therapy reduced clinical and endoscopic disease activity in patients with early endoscopic recurrence after surgery.

Our infliximab therapy was started at 6 mo after surgery when endoscopic recurrence was confirmed[20]. However, our clinical and endoscopic disease activity at 1 year after surgery was similar to that in Regueiro's study[18]. These results indicate that a 6-mo postoperative observation period does not adversely affect the efficacy of infliximab. It is reasonable to identify patients who require infliximab treatment according to endoscopic findings at 6 mo after surgery.

Sorrentino *et al*[21] studied 12 consecutive patients who maintained clinical and endoscopic remission with maintenance infliximab (5 mg/kg) for 24 mo after surgery, and whose infliximab treatment was discontinued. At 4 mo after discontinuation of infliximab, 10 of the 12 patients (83%) developed endoscopic recurrence (Rutgeerts score of i2, i3, or i3). The 10 patients were treated again with infliximab (3 mg/kg, every 8 wk), and then mucosal integrity was restored and maintained for 1 year. From their findings, long-term maintenance therapy with infliximab is required to maintain mucosal integrity in patients after surgery for CD. However, a dose of 3 mg/kg (a 40% reduction from the standard dose) was sufficient to avoid endoscopic recurrence.

Management strategy for prevention of postoperative recurrence

Our management strategy for the prevention of recur-

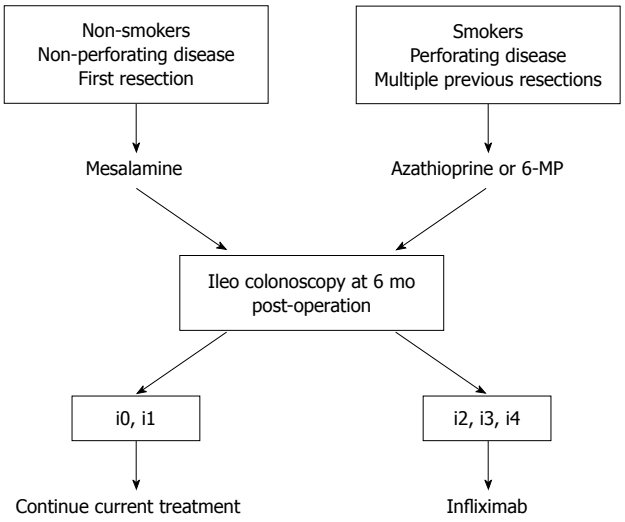

Figure 2 Algorithm for prophylactic medical treatment after ileocolonic resection in patients with Crohn's disease. 6-MP: 6-mercaptopurine.

rence after ileocolonic resection is presented in Figure 2. As a Cochrane review[12] has shown that azathioprine and 6-MP are effective for preventing post-operative recurrence, for patients with known risk factors for recurrence such as smoking, perforating disease, and multiple previous resections, treatment with azathioprine or 6-MP should be started soon after surgery. Patients without these risk factors are treated with mesalamine. At 6 mo after surgery, ileo colonoscopy should be conducted in all patients, and the severity of macroscopic inflammation in the neo-terminal ileum assessed. In patients with no or mild endoscopic lesions (Rutgeerts score of i0 or i1), current management can be continued. However, we recommend repeat endoscopic examination 6 mo later for early detection of endoscopic lesions. For patients who develop early endoscopic lesions (Rutgeerts score of i2, i3, or i4) at 6 mo after surgery despite optimal mesalamine or immunosuppressant therapy, infliximab treatment should be considered.

CONCLUSION

Infliximab therapy showed clear suppressive effects on clinical and endoscopic disease activity in patients with early endoscopic lesions after ileocolonic resection for CD. These data strongly suggest that infliximab can delay the timing of postoperative recurrence and reduce recurrence rates by improving endoscopic inflammation. Patients with early endoscopic lesions are good candidates for infliximab therapy after surgery. However, RCTs with a larger number of patients and a longer duration of follow-up are necessary to more accurately evaluate the efficacy of infliximab in early endoscopic lesions after surgery for CD.

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REVIEW

Anatomy of the lateral ligaments of the rectum: A controversial point of view

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Abstract

The existence and composition of the lateral ligaments of the rectum (LLR) are still the subjects of anatomical confusion and surgical misconception up to now. Since Miles proposed abdominoperineal excision as radical surgery for rectal cancer, the identification by "hooking them on the finger" has been accepted by many surgeons with no doubt; clamping, dividing and ligating are considered to be essential procedures in mobilization of the rectum in many surgical textbooks. But in cadaveric studies, many anatomists could not find LLR described by the textbooks, and more and more surgeons also failed to find LLR during the proctectomy according to the principle of total mesorectal excision. The anatomy of LLR has diverse descriptions in literatures. According to our clinical observations, the traditional anatomical structures of LLR do exist; LLR are constant dense connective bundles which are located in either lateral side of the lower part of the rectum, run between rectal visceral fascia and pelvic parietal fascia above the levator ani, and covered by superior fascia of pelvic diaphragm. They are pathways of blood vessels and nerve fibers toward the rectum and lymphatic vessels from the lower rectum toward the iliac lymph nodes.

Key words: The lateral ligaments of the rectum; Anatomy; Total mesorectal excision; Abdominoperineal excision; Fascia

Peer reviewer: Damian Casadesus Rodriguez, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

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INTRODUCTION

Surgical approaches in the treatment of rectal cancer have undergone great changes over the past decades. Technical aspects have been studied and reviewed extensively in an attempt to reduce local recurrences and to decrease the incidence of urinary and sexual morbidity, but the existence and composition of the lateral ligaments of the rectum (LLR) are still the subjects of anatomical confusion and surgical misconception up to now[1,2].

Since Miles proposed abdominoperineal excision (APR) as radical surgery for rectal cancer in 1908, APR has been rapidly accepted as a standard surgical strategy for rectal cancer[3]. Since then, most colorectal surgeons accepted that LLR is the pathway of blood vessels and nerve fibers toward the rectum and lymphatic vessels from the lower rectum toward the iliac lymph nodes, and clamping, dividing and ligating LLR are standard and indispensable procedures during APR, which are described in classical surgical textbooks[4-6].

Interestingly, based on cadaveric studies, many anatomists have intensively investigated the anatomy of LLR, but they could not find LLR described in the classical

surgical textbooks[7-10]. In 1982, Heald *et al*[11] demonstrated that total mesorectal excision (TME) alone could lead to a low rate of recurrence of rectal cancer in the pelvis and a high disease-free survival rate. But Heald and others described the sharp dissection of TME under direct view but did not mention LLR at all[12-16]. This phenomenon seemingly testified many anatomists' findings based on cadaveric studies, but many surgeons still remain with confusion and misconception about the anatomy of LLR.

CLINICAL VIEWS ABOUT THE ANATOMY OF LLR

In the past decades, the surgical approach to the treatment of rectal cancer has been greatly refined, and from a gross, blunt, and blind dissection with flush clamping of lateral expansions, it has become a more accurate and less radical procedure. All of these improvements are related to a better understanding and a wider knowledge of the clinical anatomy of the pelvis[17-24]. But there are different interpretations, and clinical studies about the anatomy of LLR still present quite diverse, and sometimes contradictory descriptions[25-28]. The existence and composition of LLR are also issues with considerable controversies[29-31].

In the history of radical surgery of rectal cancer, APR, proposed by Miles in 1908, was undoubtedly a breakthrough which greatly improved the outcomes of rectal cancer treatment[32]. He had already referred to LLR while explaining his dissection procedure, stating that the dissection is carried downward on either side until the upper surface of the levator ani muscles is reached. LLR, which is recognized as a firm vertical band of fascia, requires dissection with scissors[33]. He described that, LLR consists, on either side, of a broad band of dense connective tissues, which passes outward from the lateral walls of the rectum toward the base of the bladder at the point where the ureters terminate[34]. Afterwards, APR was rapidly accepted as a standard surgical strategy for middle and lower rectal cancer.

Goligher *et al*[35] also recognized LLR in a process of dissection around the rectum. LLR appears lateral to the mid-rectum after dissection on the anterior and posterior sides of the rectum is completed. He stressed that the lateral ligament can be clamped between the middle and index fingers of the left hand and then sharply severed. Based on Miles description and many surgeons' clinical experiences, LLR is considered to be a definitely existing anatomical structure, which is the pathway of blood vessels and nerve fibers toward the rectum and lymphatic vessels from the lower rectum toward the iliac lymph node, and clamping, dividing and ligating are required for LLR during APR. For a long time, there have been no controversies about LLR among surgeons.

The technique of TME advocated by Heald *et al*[12] in 1982 is another breakthrough of radical surgery of rectal cancer, which could lead to an amazing low rate of recurrence of rectal cancer in the pelvis and a high disease-free survival rate. Since then, TME has become a basic principle in radical surgery of rectal cancer, and anterior resection (AR) has gradually accepted to be a main surgical therapeutic strategy[36-38]. But in their articles, they did not mention LLR at all, neither did they do so in the subsequent works. And no LLR was mentioned by many other surgeons in their descriptions of mesorectal dissection. With the principle of TME being accepted, LLR seems to be absent in proctectomy[39,40]. Presently, LLR has become almost mythical structures, and met with considerable controversies among many colorectal surgeons about its existence and composition.

CONTROVERSIAL VIEWS OF LLR AMONG ANATOMISTS

With the development of radical surgery of rectal cancer, many anatomists have been engaged in the study of the anatomy of LLR. In contradiction to the classical knowledge of LLR, most anatomists studying cadavers did not find the typical structures of LLR described in traditional surgical textbooks. Their interpretations about the anatomy of LLR are quite different. The controversy focuses on three aspects: uncertainty of the existence, confusion of the composition, and unclear anatomic position in the pelvic cavity.

Jones *et al*[41] noticed that, before TME principle was wildly applied in radical surgery of rectal cancer, identification of LLR is "hooking it on the finger" by surgeons during operation, and clamping, dividing and ligating are indispensable procedures described in surgical textbooks. In contrast, the mesorectum can be dissected by either diathermy or sharp dissection alone. In order to clarify the anatomic misconception about LLR, Jones *et al*[41] studied the anatomy of LLR according to the TME principle for embalmed pelvis. In 1998, in their study of the anatomy of LLR, they concluded that, LLR does not exist; there is no anatomical argument against sharp dissection in the mesorectal plane and as a rule, there is no vessel that requires clamping; and by "hooking the finger" into the tissue literal to the rectum, it may be that the surgeon encounters mesorectal vessels and creates an artefactual ligament. This obviously raises the concern that such blunt dissection results in mesorectal tissue being left behind and increases the risk of local recurrence and severe autonomic nerve injury[42,43].

Interestingly, by reviewing the relative literatures about LLR, and studying fresh cadavers and embalmed pelvis, Nano *et al*[44] reported their interpretations of the anatomy of LLR in 2000, and drew the following conclusions: LLR is the extensions of the mesorecturn and must be cut at their attachment at the endopelvic fascia; LLR contains fatty tissue in communication with the mesorectal fat and possibly some vessels and nerve filaments that are of little importance; LLR at the endopelvic fascia is inserted under the urogenital bundle; the middle rectal artery runs anteriorly and inferiorly in respect to LLR; LLR can be cut at their insertion on the endopelvic fascia without injuring the urogenital nervous bundle, which, however, should be kept visible during this procedure, because it crosses the middle rectal artery and runs out behind the seminal ves-

icles; the lateral aspect of the rectum receives the lateral pedicle, which consists of the nerve fibers and the middle rectal artery[45].

In 2005, through studying the anatomy of human soft cadavers, Pak-art *et al*[46] found that, in 36 hemipelvic specimens, 18 LLRs were found on the right side of the rectum and 18 were found on the left side. The location of LLR was posterolateral to the rectum. The content of LLR consisted of loose connective tissues with cluster of small nerves. No artery was detected in all specimens. The small arterioles and venules were discovered in only four specimens. They concluded that, LLR is located at posterolateral side of the rectum. Its component is loose connective tissues containing multiple small nerves.

Recently, based on dissections of 32 formalin-preserved cadavers, Lin *et al*[47] found that LLR appeared in all 32 cadavers as a bundle of dense connective tissues traversing between rectum and visceral fascia instead of a pelvic sidewall. No substantial tissue strand except pelvic splanchnic nerves was found between visceral fascia and parietal fascia at the same level. The middle rectal artery was observed in only 18 of 64 pelvic-halves. The constant component of LLR was the rectal branches from the pelvic plexus, whereas the middle rectal artery was almost invisible in LLR. They concluded that, during total mesorectal excision, it is impossible to reveal LLR in correct surgical plane. The entire rectum may be mobilized without the need for ligating the middle rectal artery. The clinical significance of LLR is that, during lateral dissection, if LLR is identified, the surgical plane is medial to the visceral fascia, thus the incorrect surgical plane appears.

Obviously, these diverse descriptions and interpretations of the anatomy of LLR by the anatomists inevitably convey confusion and misconception to clinical colorectal surgeons. Meanwhile, their studies undoubtedly contribute to reveal the true nature of the anatomy of LLR.

OUR PERSPECTIVE OF THE ANATOMY OF LLR

According to our clinical observations based on hundreds of cases of AR and APR per year, the anatomical structures of LLR described by Miles and Goligher *et al* do exist, which were repeatedly testified by colorectal surgeons who performed traditional APR[48-51] (Figure 1). Because their descriptions of the anatomy of LLR were entirely based on clinical experience, and they had no idea about the concept of inter-fasciale at their time, during their blind and blunt surgical procedures, they failed to describe the precise anatomical position of LLR[52]. In fact, the structures of LLR are entirely covered by endopelvic fascia according to the modern anatomical point of view[53-57]. In other words, they are outside inter-fasciale, which is a correct surgical plane according to the TME principle. We believe that this is an important reason why LLR is rarely referred to after TME principle was adopted in radical surgery for rectal cancer.

Based on our surgical observation, we found that LLRs are connective bundles; their components include

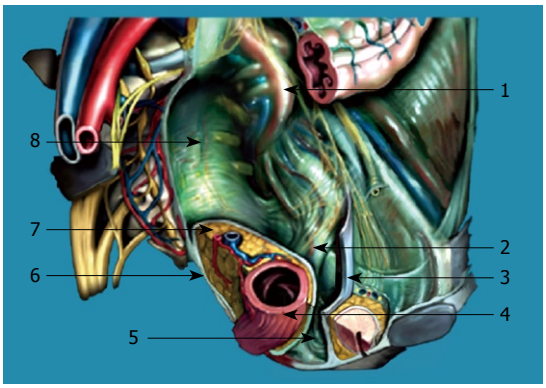

Figure 1 Illustration of the anatomy of the lateral ligament of the rectum. 1: Internal iliac artery; 2: Lateral ligament of the rectum; 3: Denonvilliers' fascia; 4: Distal part of the rectum; 5: Inter-fascial space around the distal rectum; 6: Waldeyer fascia; 7: Mesorectum; 8: Presacral fascia.

middle rectal arteries from internal iliac arteries, the rectal branches from the pelvic plexus, lymphatic vessels, some soft connective tissues and endopelvic fascia; they run between rectal visceral fascia and pelvic sidewall parietal fascia, covered by superior fascia of pelvic diaphragm above the levator ani, and terminate into the base of the distal part of the rectum laterally. Thus, LLR is a constant anatomical structure, which is the pathway of blood vessels and nerve fibers toward the rectum and lymphatic vessels from the lower rectum toward the iliac lymph nodes. The position of LLR is much lower than the surgeons thought to be. Presently, AR has been accepted as a main surgical therapeutic strategy for rectal cancer, LLR seldom needs to be treated during operation. We believe this is another important reason why LLR is rarely described by modern colorectal surgeons.

According to our clinical observations, the rectal visceral fascia extends along the pelvic cavity in the ventrodorsal direction, forming a continuous "hammock-like" sheath, enveloping the rectum[58,59]. Inside the inter-fasciale between rectal visceral and pelvic parietal fascia, there is a continuous soft connective tissue layer which is a potential surgical plane containing no real ligation structures (Figure 2). These anatomical observations were testified by the study of Jones *et al*[42]. And at the middle part of the rectum, LLR described by Nano *et al*[44] is actually artifacts due to not strictly mobilizing the rectum along inter-fasciale between visceral and parietal fascia. But at the lower part of the rectum near the pelvic floor, either side of the rectum receives the lateral pedicle, which consists of nerve fibers and the middle rectal artery (Figure 3). In fact, what they called the lateral pedicle of the rectum is the real LLR described in classical surgical textbooks. Up to now, most anatomists do not acknowledge that LLR can be hooked by the finger of traditional colorectal surgeons. We believe that the real reason is that, LLR described in classical surgical textbooks is located away from where the anatomists are looking for. When they look for some structures without clear location, they may either see nothing, or mistakenly recognize other things as the structures they have already known.

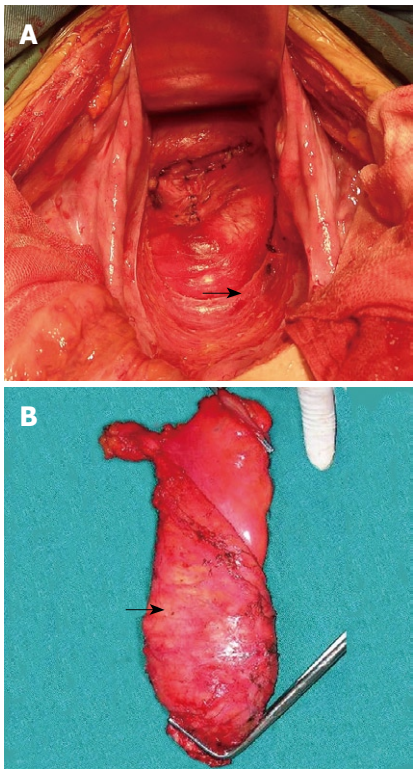

Figure 2 Operative photographs of open total mesorectal excision of rectal cancer. A: Under open surgery view, no ligament structures can be revealed in a correct surgical plane. Arrow indicates parietal pelvic fascia; B: Rectum has been removed completely. Arrow indicates rectum visceral fascia, and the rectum visceral fascia extends around the rectum, forming a continuous "hammock-like" sheath.

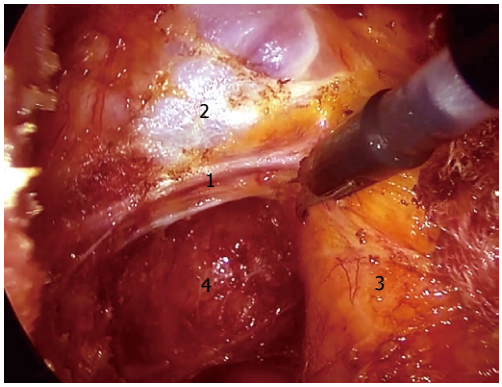

Figure 3 Operative photograph of laparoscopic total mesorectal excision of rectal cancer. 1: Left lateral ligament of the rectum; 2: Superior fascia of pelvic diaphragm; 3: Rectum visceral fascia; 4: Levator ani.

CONCLUSION

The existence and composition of LLR are still issues with considerable controversies up to now. Based on our surgical observations, we conclude that, LLRs are constant anatomical structures, which are pathways of blood vessels and nerve fibers toward the rectum and lymphatic vessels from the lower rectum toward the iliac lymph nodes; their components include middle rectal arteries, the rectal branches from the pelvic plexus, lymphatic vessels, some soft connective tissues and endopelvic fascia; their positions are at lateral to either side of the lower part of the rectum; they run between rectal visceral fascia and pelvic parietal fascia, covered by superior fascia of pelvic diaphragm above the levator ani, and terminate into the base of the distal part of the rectum laterally. From these observations, we deduce that, during total mesorectal excision, it is difficult to reveal LLR in a correct surgical plane; the entire rectum may be mobilized between visceral and parietal fascia without the need for ligating LLR; and in the process of AR and APR, we should protect the integrity of rectal visceral fascia and pelvic parietal fascia to avoid the risk of local recurrence and severe autonomic nerve injury[60-62].

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ORIGINAL ARTICLE

Monitoring early responses to irradiation with dual-tracer micro-PET in dual-tumor bearing mice

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Author contributions: Wang H and Liu B contributed equally to this work; Wang H and Liu B performed the majority of experiments; Xu BX, Guan ZW, Liu CB, Wang RM collected all the PET imaging material; Chen YM, Zhang JM coordinated and provided vital reagents and analytical tools; Qu BL performed X-ray irradiation; Tian JH prepared and edited the manuscript.

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Abstract

AIM: To monitor the early responses to irradiation in primary and metastatic colorectal cancer (CRC) with ^{18}F-fluorothymidine (^{18}F-FLT) and ^{18}F-fluorodeoxyglucose (^{18}F-FDG) small-animal position emission tomography (micro-PET).

METHODS: The primary and metastatic CRC cell lines, SW480 and SW620, were irradiated with 5, 10 and 20 Gy. After 24 h, the cell cycle phases were analyzed. A dual-tumor-bearing mouse model of primary and metastatic cancer was established by injecting SW480 and SW620 cells into mice. micro-PET with ^{18}F-FLT and ^{18}F-FDG was performed before and 24 h after irradiation with 5, 10 and 20 Gy. The region of interest (ROI) was drawn over the tumor and background to calculate the ratio of tumor to non-tumor (T/NT) in tissues. Immunohistochemical assay and Western blotting were used to examine the levels of integrin β_3, Ki-67, vascular endothelial growth factor receptor 2 (VEGFR2) and heat shock protein 27 (HSP27).

RESULTS: The proportion of SW480 and SW620 cells in the G_2-M phase was decreased with an increasing radiation dose. The proportion of SW480 cells in the G_0-G_1 phase was increased from 48.33% ± 4.55% to 87.09% ± 7.43% ($P < 0.001$) and that of SW620 cells in the S-phase was elevated from 43.57% ± 2.65% to 66.59% ± 7.37% ($P = 0.021$). In micro-PET study, with increasing dose of radiation, ^{18}F-FLT uptake was significantly reduced from 3.65 ± 0.51 to 2.87 ± 0.47 ($P = 0.008$) in SW480 tumors and from 2.22 ± 0.42 to 1.76 ± 0.45 ($P = 0.026$) in SW620 tumors. ^{18}F-FDG uptake in SW480 and SW620 tumors was reduced but not significantly ($F = 0.582$, $P = 0.633$ *vs* $F = 0.273$, $P = 0.845$). Dose of radiation was negatively correlated with ^{18}F-FLT uptake in both SW480 and SW620 tumors ($r = -0.727$, $P = 0.004$; and $r = -0.664$, $P = 0.009$). No significant correlation was found between ^{18}F-FDG uptake and radiation dose in SW480 or SW620 tumors. HSP27 and integrin β_3 expression was higher in SW480 than in SW620 tumors. The T/NT ratio for ^{18}F-FLT uptake was positively correlated with HSP27 and integrin β_3 expression ($r = 0.924$, $P = 0.004$; and $r = 0.813$, $P = 0.025$).

CONCLUSION: ^{18}F-FLT is more suitable than ^{18}F-FDG in monitoring early responses to irradiation in both primary and metastatic lesions of colorectal cancer.

Key words: ^{18}F-fluorothymidine; ^{18}F-fluorodeoxyglucose; Irradiation; Positron emission tomography; Colorectal cancer

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Wang H, Liu B, Tian JH, Xu BX, Guan ZW, Qu BL, Liu CB, Wang RM, Chen YM, Zhang JM. Monitoring early responses to irradiation with dual-tracer micro-PET in dual-tumor bearing mice. *World J Gastroenterol* 2010; 16(43): 5416-5423 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i43/5416.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i43.5416

INTRODUCTION

Radiation therapy has long been used for curative or palliative management of colorectal cancer (CRC) patients[1,2]. However, some patients undergoing radiotherapy might present primary CRC lesions as well as metastatic lymph nodes. The current method for assessing the response of a solid tumor to radiotherapy is to measure the change in tumor size on anatomical imaging modalities. It takes weeks to months to detect the change, so it is difficult to evaluate early responses to therapy *via* morphological means. Noninvasive methods for monitoring early responses to radiotherapy would be of great value in individualized treatment.

Positron emission tomography (PET) is a quantitative molecular imaging technique that allows for noninvasive *in vivo* imaging and quantification of biological processes[3-5]. ^{18}F-fluorodeoxyglucose (^{18}F-FDG) is the most widely used PET tracer and has become an indispensable staging modality for many types of cancer. However, ^{18}F-FDG may be unsuitable for monitoring the response after radiotherapy, because increased uptake can appear in inflammatory lesions and fibrosis[6-8].

^{18}F-fluorothymidine (^{18}F-FLT) is a pyrimidine analogue that uses the salvage pathway of DNA synthesis to reveal cell proliferation. ^{18}F-FLT has been found useful for noninvasive assessment of the proliferation rate of several types of cancer, such as colorectal, esophageal, breast and laryngeal cancer. Imaging and measurement of proliferation with ^{18}F-FLT-PET could be a noninvasive tool to monitor the response to anticancer treatment[9,10].

Recently, many studies claimed that PET has a special promise as a biomarker for anticancer treatment, and can be used longitudinally and provide information on the whole body or tumor. Early identification of cancer patients who are responding or resistant to radiotherapy may lead to individualized therapeutic approaches and improved clinical outcomes[11]. Yang *et al*[12] found that tumor uptake of ^{18}F-FLT was reduced significantly at 24 h after radiation with 10 Gy and 20 Gy compared with ^{18}F-FDG. At 48 h after irradiation, ^{18}F-FLT uptake was further reduced, but ^{18}F-FDG uptake was reduced slightly. So, ^{18}F-FLT-PET may be a promising imaging modality for monitoring the early effects of radiation therapy.

Bearing those in mind, we wondered whether ^{18}F-FLT could be used to reflect the early response to irradiation and compared ^{18}F-FLT and ^{18}F-FDG-PET in a possible early response in CRC primary or metastatic lesions. We chose two kinds of human CRC cells, SW480 and SW620[13-15], derived from CRC primary and lymph-node metastatic lesions, respectively, in the same patient to create a dual-tumor-bearing model. PET with ^{18}F-FLT and ^{18}F-F DG was performed before and 24 h after increasing doses of irradiation. The radioactivity uptake in SW480 and SW620 tumors was investigated with small-animal (micro)-PET.

MATERIALS AND METHODS

Chemicals

RPMI1640, Leibovitz's L15 medium, and fetal bovine serum (FBS) were obtained from PAA Laboratories GmbH, Linz, Austria. All the other chemicals were of reagent grade. Cell cycle and cell apoptosis kits were from Nanjing Keygen Biotechnology. Antibodies against K-i67, a cell proliferation antigen, and anti-integrin β_3 were from Santa Cruz Biotechnology. The antibodies anti-vascular endothelial growth factor receptor 2 (VEGFR2) and anti-heat shock protein (HSP) 27 were from Abcam. The diaminobenzidine (DAB) kit was obtained from Zhongshan Biotechnology Co., Beijing, China.

Cell lines

The human CRC cell lines SW480 and SW620 were from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). SW480 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (10 000 IU/mL), at 5% CO_2 in a humidified atmosphere at 37℃. SW620 cells were cultured in L15 medium supplemented with 10% FBS and 1% penicillin/streptomycin (10 000 IU/mL) without CO_2 in a humidified atmosphere at 37℃.

Animal tumor model

Eighteen male Balb/C nude mice (6 wk old, 20 g) were obtained from the Animal Laboratory of the Chinese Academy of Sciences. Two tumors per animal were generated by inoculating 5×10^6 SW480 viable cells into nude mice on the lateral side of the left front leg and the same amount of SW620 cells on the right front leg. Mice were kept under sterile conditions with a standard light/dark cycle and had free access to food and water. Tumor size in the front legs was determined by caliper measurement at least twice a week by the formula $V = 1/2 (l \times w \times h)$ (l, length; w, width; h, height of the tumor). Micro-PET/CT scans of ^{18}F-FDG and ^{18}F-FLT uptake were performed for tumors with volumes between 100 and 500 mm^3.

X-ray irradiation

Local external beam radiation was applied using a clinical X-ray therapy unit (Precise ELEKDA, 6 MV X-ray, at a dose rate of 388 MU/min). The mice were anesthetized using 1% chloral hydrate (0.45 mg/g body weight) and

positioned prone on the scanning table. The dual tumors were locally irradiated and the other parts of the mouse body were protected from irradiation with lead shielding. For homogeneous dose distributions, antero-posterior and postero-anterior external beam radiation fields were used. When SW480 and SW620 cells arrived at 50% confluence and when tumor size was about 100-500 mm^3, cells and tumors underwent single-dose irradiation at 5 Gy (n = 6 wells or mice), 10 Gy (n = 6) and 20 Gy (n = 6), respectively. After 24 h, tumors underwent micro-PET/CT scanning. All animal experiments were carried out in accordance with the Dutch Law on Animal Experimentation and approved by the institutional committee on animal experimentation of our institution.

Flow cytometric analysis of cell cycle

At 24 h after irradiation, SW480 and SW620 cells were washed twice with phosphate-buffered saline (PBS), detached with 0.25% trypsin and fixed with 75% ethanol and stored at 4℃. Cells were centrifuged to remove 75% ethanol before cell cycle determination, washed twice with PBS and resuspended in 0.5 mL PBS. After cells were stained with propidium iodide in the dark for 10 min, DNA content was measured by flow cytometry (FACScalibur, Becton Dickinson) to obtain the percentage of cells in each phase.

PET studies and image analysis

PET images of tumors in dual-tumor-bearing mice were obtained using the small animal micro-PET/CT (Explore VISTA micro-PET/CT, GE). At 24 h after irradiation, 3 mice in each group underwent ^{18}F-FLT PET and 3 mice underwent ^{18}F-FDG PET. The mice were anesthetized and positioned prone in the scanner, and ^{18}F-FLT or ^{18}F-FDG was injected *via* the tail vein at 20 ± 1.84 MBq in 0.25 mL saline. Image data were acquired for 10 min at 1 h after injection. For image reconstruction, list-mode data were sorted into 3-D sinograms, then underwent Fourier rebinning and 2-D ordered-subset expectation maximization reconstruction with 2 iterations and 50 subsets. Image pixel size was 0.385 mm × 0.385 mm × 0.335 mm. For quantitation of tumor uptake of ^{18}F-FLT or ^{18}F-FDG, image software was used to analyze the region of interest (ROI) in reconstructed images. Three consecutive coronal slice images containing tumors were selected visually, and ROIs were drawn on the tumor and lung as background, and the ratio of tumor to background (T/NT) uptake was calculated.

Immunohistochemical and Western blotting analysis

The staining procedure has been described elsewhere[10]. Sections of two kinds of tumors were stained with hematoxylin and eosin and antibodies against anti-integrin β3 (sc-52685), anti-Ki67 (sc-52685), anti-HSP27 (ab2790), and anti-VEGFR2 (ab3968) antibody (1:100). For a negative control, the primary antibody was omitted and replaced with PBS. Specimens were examined under light microscopy. The number of integrin β3-, Ki-67-, HSP27-, and VEGFR2-positive and HE-positive cells in adjacent sections was counted in 5 randomly selected fields per section. Western blotting analysis of 150 μg protein from SW480 and SW620 tumors was performed as described earlier[15]. The antibodies were the same as described in the immunocytochemical assay. Integrin β3, Ki-67, HSP27, and VEGFR2 expression was described by gray scale analysis with the Labworks software.

Statistical analysis

Data analysis was performed using SPSS v11.5 (SPSS Inc., Chicago, IL). Percentages of cells in each cell phase after irradiation were compared by one-way ANOVA. Differences in radiotracer uptake before and after irradiation in each mouse were compared by independent-samples t test. Linear regression analysis was used to determine the correlation between radiotracer uptake and radiation dose or cell cycle phase. All data were expressed as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Cell cycle analysis

We examined the effects of irradiation on cell cycle distribution by flow cytometry. At 24 h after irradiation, the proportion of SW480 cells in the G_0-G_1 phase decreased from 48.33% ± 4.55% at 0 Gy to 26.70% ± 7.09% at 5 Gy, then increased to 87.09% ± 7.43% at 20 Gy, the proportion in the S phase decreased from 33.23% ± 6.09% at 0 Gy to 12.44% ± 4.60% at 20 Gy; and that in the G_2-M phase decreased from 18.44% ± 5.67% at 0 Gy to 0.47% ± 0.34% at 20 Gy (Figure 1A). At 0-20 Gy, the proportion of SW620 cells in the G_0-G_1 phase decreased from 39.37% ± 4.37% to 20.39% ± 5.12%, and that in the S phase increased from 43.57% ± 2.65% to 66.59% ± 7.37%. The proportion in G_2-M phase decreased from 17.07% ± 3.09% to 13.02% ± 4.55% (Figure 1B).

Micro-PET/CT analysis

Micro-PET/CT scanning results of ^{18}F-FLT and ^{18}F-FDG uptake in SW480 and SW620 tumors irradiated with increasing doses are shown in Table 1 and Figure 2. Before irradiation, the T/NT ratio in the ROI for ^{18}F-FLT was higher in SW480 (3.65 ± 0.51) than in SW620 tumors (2.22 ± 0.42). At 24 h after irradiation with 20 Gy, the T/NT ratio for ^{18}F-FLT uptake was significantly decreased in both SW480 (2.87 ± 0.47, P = 0.008) and SW620 cells (1.76 ± 0.45, P = 0.026) (Figure 2A).

Before irradiation, the T/NT ratio for ^{18}F-FDG in SW480 and SW620 tumors was 2.69 ± 0.98 and 3.09 ± 1.26, respectively (P = 0.524). At 24 h after irradiation at 20 Gy, the T/NT ratio for ^{18}F-FDG uptake was reduced but not significantly (2.40 ± 0.52 and 2.89 ± 0.29, both $P > 0.05$) (Figure 2B).

Immunohistochemical and Western blotting analysis

Integrin β3, HSP27, Ki-67 and VEGFR2 proteins were all overexpressed in SW480 and SW620 tumors (Figure

Table 1 Ratio of tumor to non-tumor for ^{18}F-fluorothymidine and ^{18}F-fluorodeoxyglucose uptake in SW480 and SW620 tumors in mice (mean ± SD)

Tracer	SW480				SW620			
	0 Gy	5 Gy	10 Gy	20 Gy	0 Gy	5 Gy	10 Gy	20 Gy
^{18}F-FLT	3.65 ± 0.51	3.75 ± 0.71	3.04 ± 0.35	2.87 ± 0.47[b]	2.22 ± 0.42	2.47 ± 0.59	2.10 ± 0.55	1.76 ± 0.45[a]
^{18}F-FDG	2.69 ± 0.98	2.85 ± 0.47	2.62 ± 0.67	2.40 ± 0.52	3.08 ± 1.26	2.92 ± 0.42	3.22 ± 0.56	2.89 ± 0.29

[a]$P < 0.05$, [b]$P < 0.01$, 3 mice/group. ^{18}F-FLT: ^{18}F-fluorothymidine; ^{18}F-FDG: ^{18}F-fluorodeoxyglucose.

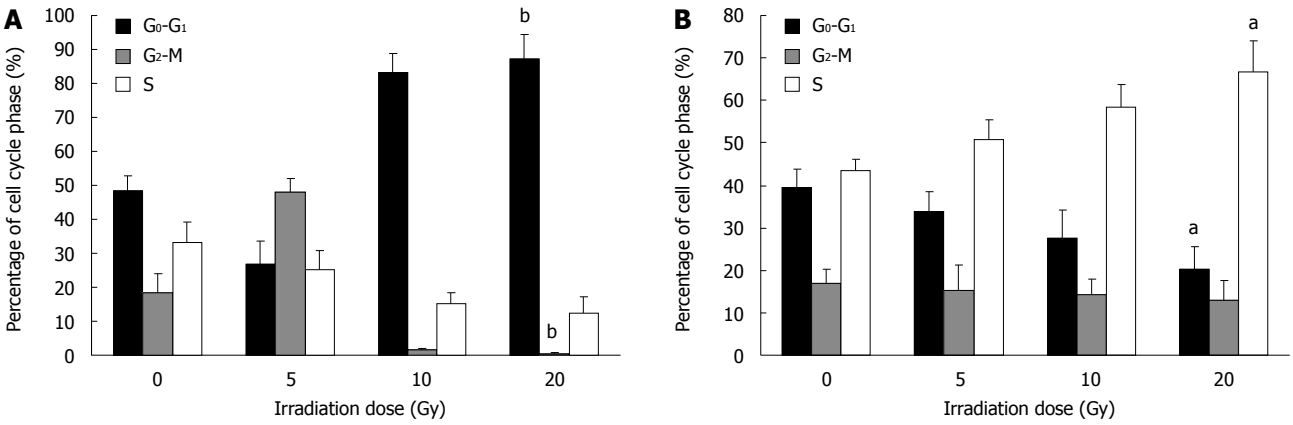

Figure 1 Flow cytometry of cell cycle response to irradiation in SW480 (A) and SW620 (B) cells. [a]$P < 0.05$, [b]$P < 0.01$.

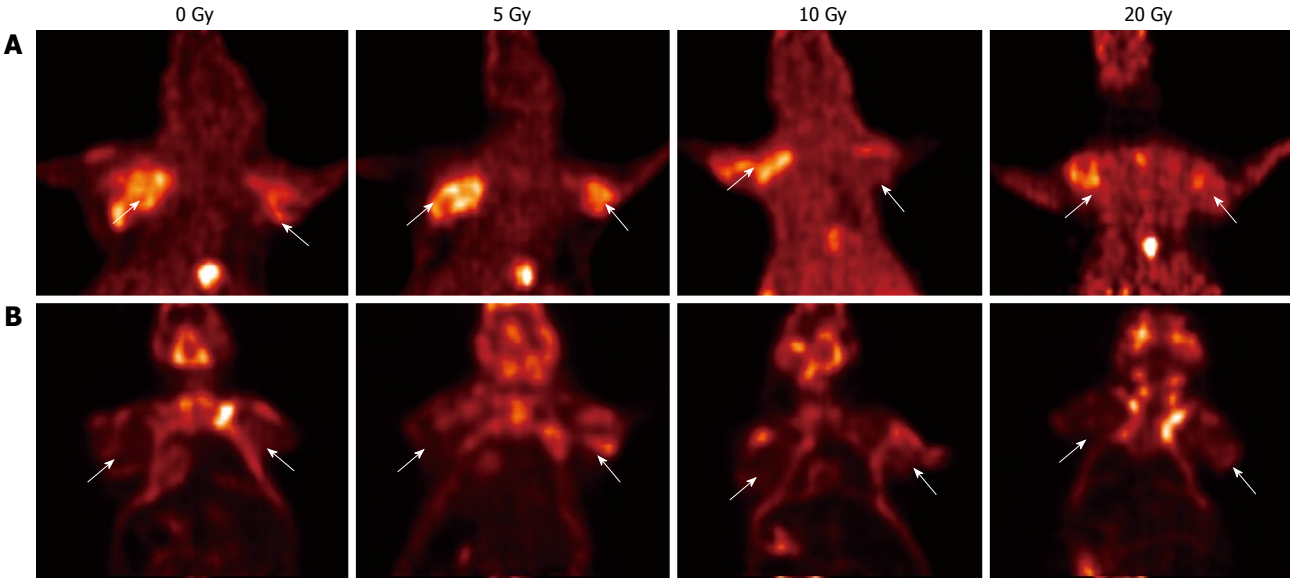

Figure 2 Coronal micro-position emission tomography sections of mice 60 min after injection with ^{18}F-fluorothymidine (A) or ^{18}F-fluorodeoxyglucose (B) before and 24 h after irradiation (3 mice/ group). Implanted tumors (arrows) are located on the left (SW480) and the right (SW620) front legs of mice. Uptake of radiotracers was normalized to lung radiotracer uptake and expressed as ratio to non-tumor (T/NT) uptake.

3). SW480 cells showed more intense staining for integrin β3 and HSP27 protein in cytoplasm or nucleus than did SW620 tumors. Integrin β3 protein was also overexpressed in the tumor matrix near vasculature. Staining for VEGFR2 and especially Ki-67 expression was lower in SW480 tumors than in SW620 tumors.

Western blotting analysis revealed that HSP27 and integrin β3 expression was higher in SW480 than in SW620 tumors (42.86% ± 5.15% *vs* 10.10% ± 3.50%, for Hsp27, $P = 0.002$; and 9.61% ± 3.20% *vs* 8.43% ± 1.85% for integrin β3, $P = 0.164$). The expression of K-i67 and VEGFR2 protein was less pronounced in SW480 than in SW620 tumors (6.5% ± 1.25% and 9.00% ± 2.38% for K-i67, $P = 0.009$; and 25.33% ± 5.59% and 19.96% ± 4.20% for VEGFR2, $P < 0.001$) (Figure 4A and B).

Linear regression analysis

We found a significant negative correlation between dose

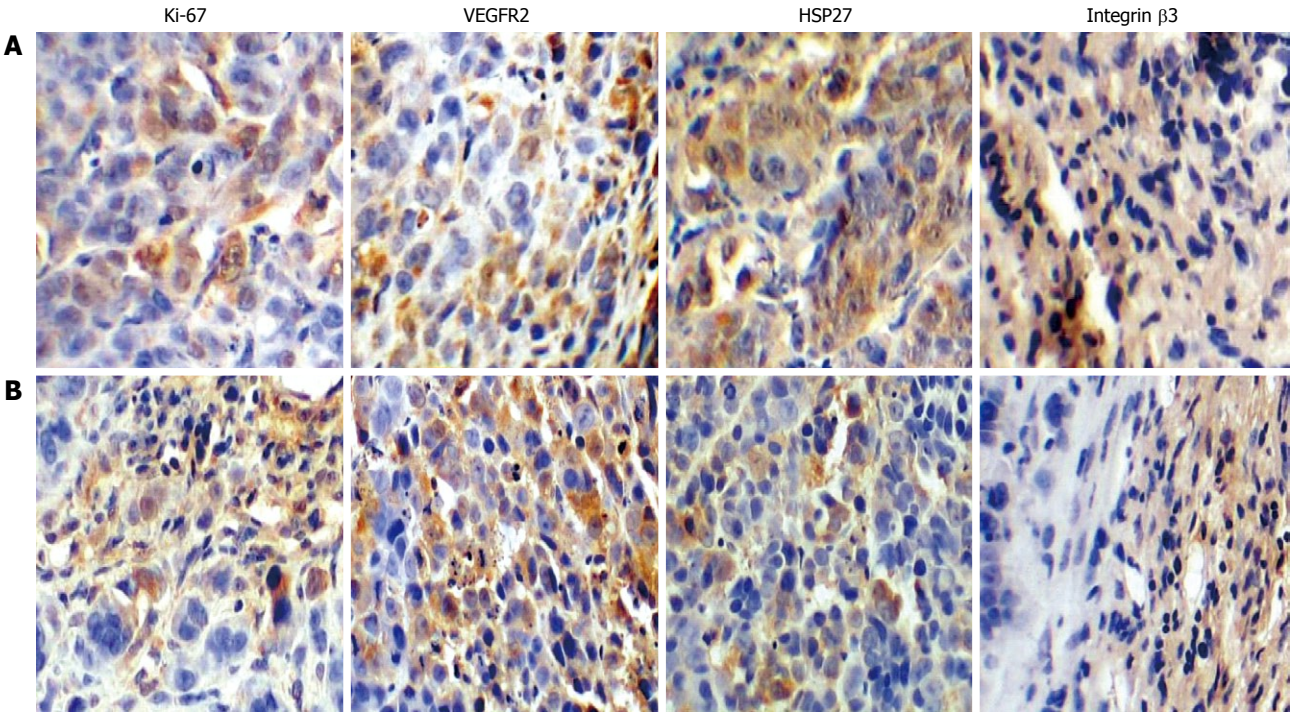

Figure 3 Immunohistochemical analysis of expression of integrin β3, K-i67, heat shock protein 27 and vascular endothelial growth factor receptor 2 protein in SW480 (A) and SW620 (B) cell lines by immunohistochemical detection (400 ×). VEGFR2: Vascular endothelial growth factor receptor 2; HSP27: Heat shock protein 27.

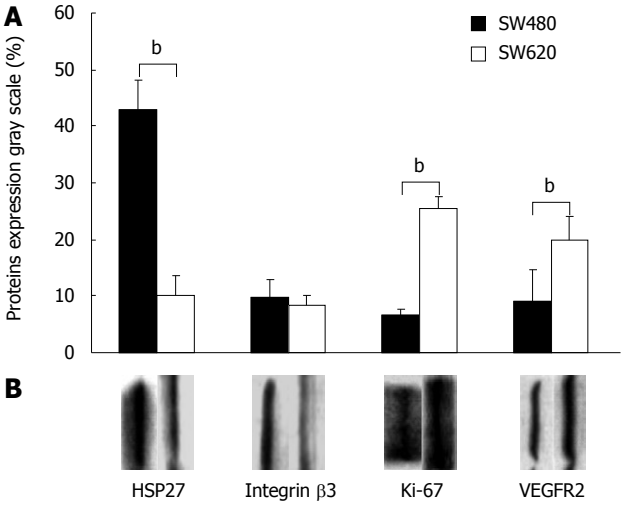

Figure 4 Western blotting analysis and quantitation of heat shock protein 27, integrin β3, Ki-67, and vascular endothelial growth factor receptor 2 protein expression in SW480 and SW620 tumors. $^{b}P < 0.01$. VEGFR2: Vascular endothelial growth factor receptor 2; HSP27: Heat shock protein 27.

of radiation and ^{18}F-FLT uptake in SW480 and SW620 tumors (r = -0.727, P = 0.004, and r = -0.664, P = 0.009, respectively). Dose of radiation was positively but not significantly correlated with ^{18}F-FDG uptake in SW480 tumors (r = 0.401, P = 0.098) and positively but significantly correlated with ^{18}F-FDG uptake in SW620 tumors (r = 0.640, P = 0.013). Dose of radiation was negatively correlated with proportion of SW480 cells in the G_2-M phase (r = -0.798, P = 0.001) and negatively but not significantly correlated with proportion of SW620 cells in the G_2-M phase (r = -0.184, P = 0.283). Dose of radiation was positively correlated with proportion of SW620 cells in the S phase (r = 0.870, $P < 0.001$) and negatively correlated with proportion of SW620 cells in the G_0-G_1 phase (r = -0.6730, P = 0.008). The T/NT ratio for ^{18}F-FLT uptake was positively correlated with integrin β_3 and HP27expression (r = 0.813, P = 0.025, and r = 0.924, P = 0.004), but not with Ki-67 or VEGFR2 expression. Similarly, the T/NT ratio for ^{18}F-FDG uptake was not significantly correlated with integrin β_3, HSP27, Ki-67 and or VEGFR2 expression.

DISCUSSION

Therapy monitoring plays a major role in the evaluation of therapeutic approaches[16-18]. ^{18}F-FDG-PET is clinically used for the diagnosis, staging and re-staging of a wide variety of tumors. However, the technique contains several shortcomings in reflecting changes in tumors after treatment, especially radiotherapy[19-22]. Several research groups have suggested that ^{18}F-FLT, as a cell proliferation tracer, is a more cancer-specific tracer than ^{18}F-FDG[23-27], but some results are contradictory[28]. In the present study, we investigated ^{18}F-FLT-PET as a potential tool for monitoring the early response to irradiation in a mouse model of dual-tumor-bearing CRC. In clinical practice, we have often found primary lesions and metastatic lymph nodes together in the same patient. A model of dual tumors created with CRC SW480 and SW620 cells is similar to clinical practice, so we compared the uptake of ^{18}F-FLT and ^{18}F-FDG in response to irradiation in the two kinds of CRC tumors.

In a micro-PET study, we found a higher uptake of ^{18}F-FLT than ^{18}F-FDG in SW480 and SW620 tumors. After irradiation for 24 h, the uptake of ^{18}F-FLT in SW480 or SW620 tumors increased at a low dose (5 Gy), then reduced gradually with increasing radiation dose. A statistical difference was found in both tumor groups although ^{18}F-FLT uptake reduced more significantly in SW480. Whereas the ^{18}F-FDG uptake was increased at a low dose (5 Gy) and reduced slightly at a high dose (20 Gy) without a significant difference. Liang *et al*[13] did not find a dose-dependent decrease in uptake of ^{18}F-FLT in tumors. However, in the current study, with a dose greater than 5 Gy, the T/NT ratio for ^{18}F-FLT uptake was dose-dependently decreased in both SW480 and SW620 tumors. A significantly negative correlation was found between dose of radiation and ^{18}F-FLT uptake in SW480 and SW620 tumors. No correlation was found between dose of radiation and ^{18}F-FDG uptake. So ^{18}F-FLT uptake can be more sensitive and accurate than ^{18}F-FDG to monitor the response to irradiation after 24 h.

Recent studies have shown that a decrease in cellular proliferation rate is one of the early events in response to tumor treatment. In the present study, after 24 h irradiation, cell cycle redistribution was found, and proliferation inhibition of the two kinds of CRC cells occurred in a dose-dependent manner, and the response to seam dose was different. G_2-M phase decrease and G0-G1 phase arrest were found earlier in SW480 than that in SW620 cells. We also found a decrease in the proportion of SW480 and SW620 cells in the S phase with the increasing radiation dose, and the dose of irradiation was negatively correlated with proportion of G_2-M phase in both kinds of cells. T/NT ratio for ^{18}F-FLT was negatively correlated with proportion of SW480 or SW620 cells in the G_2-M phase. The proportion of cells decreased in the G_0-G_1 arrest and S phases may not be important for the ^{18}F-FLT uptake decrease in SW480 and SW620 tumors.

A previous study with another tumor cell line also showed that tumor uptake of ^{18}F-FDG was decreased at 24 h after irradiation[29]. The mechanism of the increased ^{18}F-FDG and ^{18}F-FLT uptake after 24 h irradiation at a low dose (5Gy) has remained unclear. It may be due to the G_2-M or S phase arrest enhancing metabolism after irradiation in a short time. After 24 h irradiation, the two kinds of tumors were excised immediately. No necrosis formation occurred in the tumors possibly due to the short time (24 h) after radiotherapy.

In our dual-tumor-bearing animal study, the reason for the difference in ^{18}F-FLT and ^{18}F-FDG uptake in response to irradiation is not clear. The different response may be related to different biological characteristics, such as tumor marker expression. A large number of different proteins are expressed in SW480 and SW620 cell lines. In this study, we selected 4 tumor biomarkers, integrin β_3, Ki67, HSP27 and VEGFR2, known as tumor invasion, proliferation, apoptosis, angiogenesis markers, respectively[30-32]. VEGFR2 or Ki-67 expression was stronger in SW620 than in SW480 cells. In contrast, HSP27 and integrin β_3 expression was more intense in SW480 cells. The T/NT ratio for ^{18}F-FLT was significantly correlated with HSP27 and integrin β_3 expression. Tumor biomarkers play a major role in indicating tumor characteristics, exploring possible mechanisms, and in evaluating and suggesting new therapeutic approaches. The results suggest that other factors, besides proliferation, may influence the response to irradiation.

Although our study population is small, differences were found in the ^{18}F-FLT and ^{18}F-FDG uptake response in CRC to radiotherapy on PET. ^{18}F-FLT PET may be a useful noninvasive imaging modality to assess early response to irradiation in both primary and metastatic CRC lesions. ^{18}F-FLT-PET is also quick and effective in monitoring the response to irradiation in primary tumors. The capacity of ^{18}F-FLT to reveal response to irradiation within 24 h may be useful for individualizing therapy. Additional studies are being carried out to investigate other radiation doses and the mechanism among different tumor cells.

In conclusion, compared with ^{18}F-FDG-PET, ^{18}F-FLT-PET might be better in monitoring the response to 24 h irradiation in both primary and metastatic CRC lesions with increasing radiation doses.

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COMMENTS

Background

Colorectal cancer is one of the most frequently encountered malignancies in China and is associated with a high mortality rate. Irradiation therapy has long been used for curative or palliative management in colorectal cancer (CRC). When irradiation was performed, primary CRC and metastatic lymph node metastatic lesions often appear in the same patient.As it takes weeks to months to detect the change, it is difficult to evaluate the early responses to therapy *via* morphological means. Noninvasive methods for monitoring early responses to radiotherapy would be of great value in individualized treatment. So there are some questions to answer: Weather ^{18}F-fluorodeoxyglucose (^{18}F-FDG) or ^{18}F-fluorothymidine (^{18}F-FLT) could depict the difference earlier, and which is more suitable for doing so? This study was designed to investigate whether ^{18}F-FLT-PET could be used to reflect the early effect of irradiation in both CRC primary and lymph node metastatic lesions as compared with ^{18}F-FDG-PET.

Research frontiers

Recently, many studies claimed that PET has particular promise as a biomarker for anticancer therapies, can be used longitudinally and provides information on the patient or tumor. Early identification of cancer patients who are responding or resistant to radiotherapy may lead to individualized therapeutic approaches and improved clinical outcomes. ^{18}F-FLT-PET may be a promising imaging modality for monitoring the early effects of radiation therapy.

Innovations and breakthroughs

^{18}F-FLT PET may be used as a useful noninvasive imaging modality to monitor early response to irradiation for different CRCs. This is the first study to report in a new angle that ^{18}F-FLT is more helpful than ^{18}F-FDG in reflecting the early effects of irradiation in CRC primary lesions or lymph metastatic lesions.

Applications

SW480 and SW620 tumors, either primary or from metastatic lymph nodes have different responses to irradiation at early phase; ^{18}F-FLT response to irradiation is more sensitive than ^{18}F-FDG. Evaluation of the response to irradiation would be helpful for individualizing treatment and improving outcomes of CRC patients in clinical practice.

Terminology

^{18}F-FDG is the most widely used PET tracer, but it has several shortcomings in reflecting changes in tumors after treatment, especially radiotherapy. ^{18}F-FLT is a pyrimidine analogue and believed to be an agent for imaging cellular proliferation via the salvage pathway of DNA synthesis, which is closely associated with cellular proliferation. The 4 kinds of tumor biomarkers, HSP27, Integrinβ_3, VEGFR2 and Ki67, are related to tumor differentiation, invasion, angiogenesis and proliferation respectively.

Peer review

The authors investigated whether ^{18}F-FLT-PET or ^{18}F-FDG-PET could be used to reflect the early effects of irradiation in CRC primary lesions or lymph metastatic lesions. The results revealed that ^{18}F-FLT-PET might be better in monitoring the response to 24 h irradiation in both primary and metastatic CRC lesions with increasing radiation doses. The results are interesting and helpful for individualized treatment in clinical practice.

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ORIGINAL ARTICLE

Endogenous leptin fluctuates in hepatic ischemia/reperfusion injury and represents a potential therapeutic target

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Abstract

AIM: To evaluate the role of leptin in the internal disorders during hepatic ischemia/reperfusion injury.

METHODS: A rat model of 70% hepatic ischemia/reperfusion injury was established, with groups of sham-operation (Sham), 60 min ischemia/60 min reperfusion (I60′R60′), I60′R150′, I60′R240′ and I60′R360′. Serum leptin was detected by a self-produced radioimmunoassay; serum glucose, total anti-oxidation capacity, myeloperoxidase, alanine transaminase and diamine oxidase were determined by relevant kits, while histological alterations and protein levels of leptin in the lung, liver and duodenum were examined by hematoxylin-eosin staining and immunohistochemistry. Spearman's rank correlation between leptin and other variables or grading of tissue impairment were analyzed simultaneously.

RESULTS: Serum leptin in I60′R360′ was significantly higher than in Sham and I60′R240′ groups (both $P < 0.05$), serum glucose in I60′R360′ was higher than in Sham and I60′R150′ (both $P < 0.05$), and serum total anti-oxidation capacity in I60′R240′ and I60′R360′ were higher than in Sham (both $P < 0.05$) and I60′R150′ groups (both $P < 0.01$). Serum myeloperoxidase in groups of I60′R240′ and I60′R360′ were lower than in I60′R150′group (both $P < 0.05$), serum alanine transaminase in the four reperfusion groups were higher than in the Sham group (all $P < 0.05$), while serum DAO in I60′R360′ was lower than in I60′R60′ ($P < 0.05$). Histological impairment in the lung, liver and duodenum at the early phase of this injury was more serious, but the impairment at the later phase was lessened gradually. Protein levels of leptin in the lung in the four reperfusion groups were significantly lower than in the Sham group (all $P < 0.01$), decreasing in the order of I60′R150′, I60′R60′, I60′R360′ and I60′R240′; the levels in the liver in I60′R60′ and I60′R240′ were higher than in the Sham group (both $P < 0.01$), while the levels in I60′R240′ and I60′R360′ were lower than in I60′R60′ (both $P < 0.01$); the levels in duodenum in I60′R240′ and I60′R360′ were higher than in Sham, I60′R60′ and I60′R150′ (all $P < 0.01$), while the level in I60′R150′ was lower than in I60′R60′ ($P < 0.05$). There was a significantly positive correlation between serum leptin and alanine transaminase ($\rho = 0.344$, $P = 0.021$), a significantly negative correlation between the protein level of leptin in the lung and its damage scores ($\rho = -0.313$, $P = 0.036$), and a significantly positive correlation between the protein level of leptin in the liver and its damage scores ($\rho = 0.297$, $P = 0.047$).

CONCLUSION: Endogenous leptin fluctuates in he-

patic ischemia/reperfusion injury, exerts a potency to rehabilitate the internal disorders and represents a potential target for supportive therapy.

Key words: Leptin; Reperfusion injury; Liver; Lung; Duodenum; Recovery of function

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INTRODUCTION

Hepatic ischemia/reperfusion injury is a complication of liver resection, transplantation and hypovolemic shock, which leads to local and systemic cellular damage as well as organ dysfunction[1]. Several lines of evidence suggest that the first consequence of hepatic ischemia/reperfusion injury is tissue hypoxia, which disturbs the intracellular energy metabolism and enzyme functions, resulting in depletion of adenosine triphosphate, accumulation of free radicals, and lesions in vital organs[2]. This injury is largely a result of an acute inflammatory cascade, and a certain endogenous network may exist to modulate the internal disorders. However, the conceivable mechanism has not been clarified.

Leptin is an *ob* gene-expressed protein mainly secreted by adipose tissues, with a primary role of inhibiting food intake, modulating weight balance and promoting energy metabolism[3]. Previous researches have revealed that leptin is a stress mediator after injuries, and it proceeds to maintain homeostasis by accelerating oxidation of glucose and fatty acids, alleviating reactive oxygen species-induced apoptosis, and ameliorating post-septic multiple organ dysfunction[4-6]. All these results suggest an important role for leptin in the recovery of hepatic ischemia/reperfusion injury, but there is no available report, which prompts us to study the fluctuation of leptin levels and its association with metabolic disorders as well as the functional and structural impairment in vital organs during this injury.

We established a rat model of 70% hepatic ischemia/reperfusion injury, checked serum leptin levels at different reperfusion time points, and detected serum glucose and total anti-oxidation capacity (TAC) for representing metabolic disorders. Serum myeloperoxidase (MPO), alanine transaminase (ALT), diamine oxidase (DAO) and histological alterations were evaluated in the functional and structural impairment in the lung, liver and duodenum. Protein levels of leptin were also examined in the distal, local and proximal vital organs mentioned above. Correlation analysis between serum leptin and other variables in serum, and the same analysis between tissue leptin and histological alterations in each organ were performed simultaneously. We aimed to find out the potential role for leptin in the recovery of internal disorders after hepatic ischemia/reperfusion injury.

MATERIALS AND METHODS

Animals and reagents

Three male New Zealand white rabbits (weight 1.5 ± 0.1 kg) and 45 male Sprague-Dawley rats (weight 250 ± 6.0 g) were supplied by the experimental animal center of our hospital. Animals were maintained in a room at 22-25℃ under a constant day/night rhythm and given food and water *ad libitum*. All animal experiments were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at our hospital.

Recombined murine leptin was purchased from PeproTech Inc. (London, UK). Complete and incomplete Freund's adjuvant was purchased from Gibco/BRL (Gaithersburg, USA). Sodium iodide ($Na^{125}I$) was purchased from Amersham Biosciences (Piscataway, USA). Immunoturbidimetric kits for serum glucose were purchased from Biosino Biotechnology and Science Inc. (Beijing, China). Colorimetric kits for serum TAC, MPO and ALT were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Spectrophotometric assay for serum DAO was established and kindly provided by Professor Jun-You Li from Burns Institute, the First Affiliated Hospital of Chinese PLA General Hospital. Biotinylated anti-rabbit IgG, peroxidase-conjugated streptavidin and diaminobenzidine chromogenic kits were purchased from Zhongshan Golden Bridge Biotechnology Co. Ltd. (Beijing, China). Other reagents were purchased locally and of analytically pure grade.

Preparation of leptin antibody and iodination of leptin

New Zealand white rabbits were immunized subcutaneously with an emulsion of 120 μg leptin in 3 mL complete Freund's adjuvant for the first injection, then with another emulsion in incomplete Freund's adjuvant for four times to attain antibody. Iodination of leptin was performed according to our previous report[7]. The ^{125}I-labeled leptin with a high specific binding rate and a low fault binding rate was taken as successful iodinated antigen. They were mixed with an equal volume of 1.5% bovine serum albumin and stored at -20℃.

Establishment of a rat model of 70% hepatic ischemia/reperfusion injury

Forty-five rats were divided randomly into five groups, including sham-operation (Sham), hepatic ischemia for 60 min/reperfusion for 60 min (I60'R60'), I60'R150', I60'R240' and I60'R360', and each group contained 9 rats.

Rats were deprived of food 12 h prior to the start of experiment, with free access to drinking water *ad libitum*.

Rats were anesthetized with pentobarbital sodium (60 mg/kg, i.p.), and their abdominal skin was sterilized. After a 2-cm midline incision was made in abdomen, the portal vein, hepatic artery and bile duct of the left and middle lobes were occluded with a microvessel clip for 60 min, and then the clip was released for reperfusion. The three remaining caudal lobes retained an intact portal/arterial blood supply and venous outflow, thus preventing intestinal venous hypertension and possible leakage of bacteria or bacterial products into the circulation that would be found in 100% hepatic ischemia/reperfusion injury[8].

The portal vein, hepatic artery and bile duct of the left and middle lobes were not occluded in the rats of Sham group. The incision was closed in layers, and resuscitation with isotonic saline (30 mL/kg, i.p.) was supplied. Then, rats were sterilized with iodophor on their suture sites and released back to cages, with free access to drinking water *ad libitum*.

Preparation of serum and tissue samples

Blood was collected from the heart of rats at different time points under anesthesia with a syringe, and serum was separated from whole blood through incubating samples in 37℃ water for 20 min and centrifuged by 3000 × *g* for 10 min at 4℃. Lung, liver and duodenum samples of each rat were harvested immediately after attaining blood samples. Each tissue sample was fixed in 40 g/L neutral phosphate-buffered formaldehyde solution and embedded in paraffin, and then serial sections (4 μm thick) were made.

Measurement of serum leptin and other variables in serum

Serum leptin was measured by a self-produced radioimmunoassay according to our previous report[7], using the leptin antibody and iodinated leptin mentioned above. Serum glucose, TAC, MPO, ALT and DAO were measured by the kits or assay according to the provided protocol of the manufacturers.

Histological evaluation

Tissue sections were stained with hematoxylin and eosin, and observed under microscope to investigate histological alterations in the lung, liver and duodenum. Nine microscopic fields of each organ in each group were randomly selected, and grading of the structural impairment in each organ was evaluated according to the criteria outlined in the reference[9].

Immunohistochemistry

Protein levels of leptin in the lung, liver and duodenum were detected by immunohistochemistry. Briefly, tissue sections were collected on 0.1% poly-lysine coated slides, deparaffinized by the xylene-ethanol sequence, rehydrated in a graded ethanol scale and in phosphate buffered saline (PBS). After prior antigen retrieval by heating in a pressure cooker in citrate buffer, the slides were incubated sequentially with the self-produced leptin antibody, biotinylated anti-rabbit IgG and peroxidase-conjugated streptavidin at 37℃ for 1 h, using a dilution of 1:50, 1:200 and 1:200 in PBS. Diaminobenzidine chromogenic kits were used to develop the color.

Negative controls were set up simultaneously by replacing the leptin antibody with PBS. The images were visualized with Leica DC300F Digital Camera Systems, and 9 microscopic fields of each organ from each group were randomly selected. Image-Pro Plus 6.0 software was used to analyze the ratio of accumulated optical density of specific positive staining/specific tissue area in each image.

Statistical analysis

Stata 7.0 software (Stata Corp, USA) was used to analyze the data. One-way analysis of variance and Student's *t* test were applied for the data including serum variables and tissue leptin. Wilcoxon signed-rank test was used for the data about grading of tissue impairment. Spearman's rank correlation was selected for correlation analysis. A *P* value of less than 0.05 was chosen as a threshold for statistical significance, and all data were shown as mean ± SD.

RESULTS

Serum levels of leptin after hepatic ischemia/reperfusion injury

Serum leptin in I60'R360' was significantly higher than in Sham and I60'R240' (t = 3.410 and 2.659, both P < 0.05), while there was no significant difference in the levels among the Sham, I60'R60', I60'R150' and I60'R240' groups, as shown in Figure 1A.

Serum levels of glucose and TAC after injury

Serum glucose in I60'R360' was significantly higher than in Sham and I60'R150' (t = 2.190 and 3.286, both P < 0.05), and it was inclined to be higher than in I60'R60' (P = 0.051). No difference was observed in the levels among Sham, I60'R60', I60'R150' and I60'R240' (Figure 1B). Serum TAC in I60'R240' and I60'R360' were significantly higher than in Sham (t = 3.050 and 2.693, both P < 0.05) and I60'R150' (t = 3.566 and 3.270, both P < 0.01), while there was no difference either between the levels in I60'R240' and I60'R360' or in those among Sham, I60'R60' and I60'R150' (Figure 1C).

Serum levels of MPO, ALT and DAO after injury

Serum MPO in the four reperfusion groups was not different from that in Sham, but the levels in I60'R240' and I60'R360' were significantly lower than in I60'R150' (t = -2.259 and -2.489, both P < 0.05), while no difference was found in the levels among I60'R60', I60'R240' and I60'R360' (Figure 1D).

Serum ALT in I60'R60', I60'R150', I60'R240' and I60'R360' were significantly higher than in Sham (t = 4.132, 5.888, 5.201 and 4.359, all P < 0.01), while there was no difference in the levels among the four reperfusion groups, as shown in Figure 1E. Serum DAO in the four

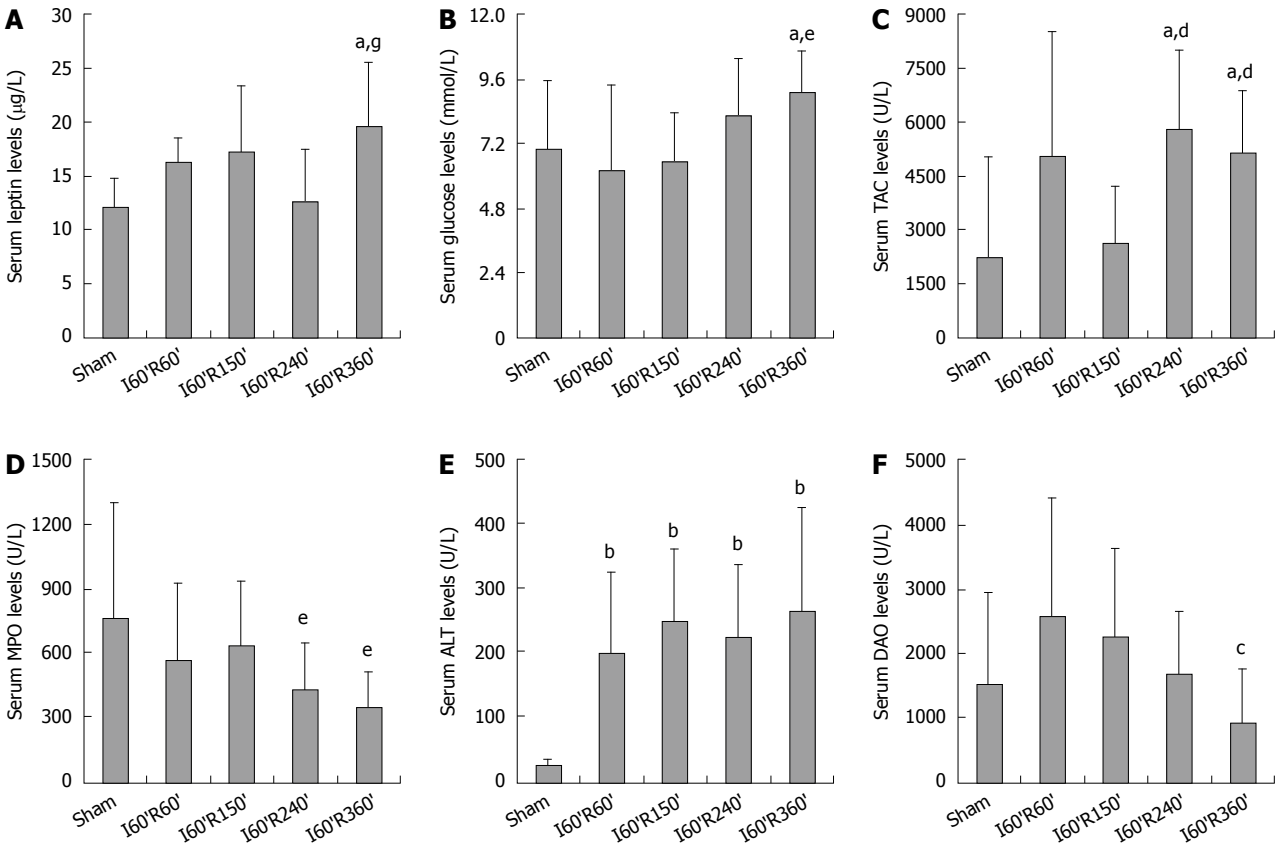


Figure 1 Serum levels of leptin (A), glucose (B), total anti-oxidation capacity (C), myeloperoxidase (D), alanine transaminase (E) and diamine oxidase (F) in the rats after injury. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* sham; $^{c}P < 0.05$ *vs* I60'R60'; $^{d}P < 0.01$, $^{e}P < 0.05$ *vs* I60'R150'; $^{g}P < 0.05$ *vs* I60'R240'. Sham: Sham-operation; I60'R60', I60'R150', I60'R240' and I60'R360': Hepatic ischemia for 60 min/reperfusion for 60, 150, 240 and 360 min; TAC: Total anti-oxidation capacity; MPO: Myeloperoxidase; ALT: Alanine transaminase; DAO: Diamine oxidase.

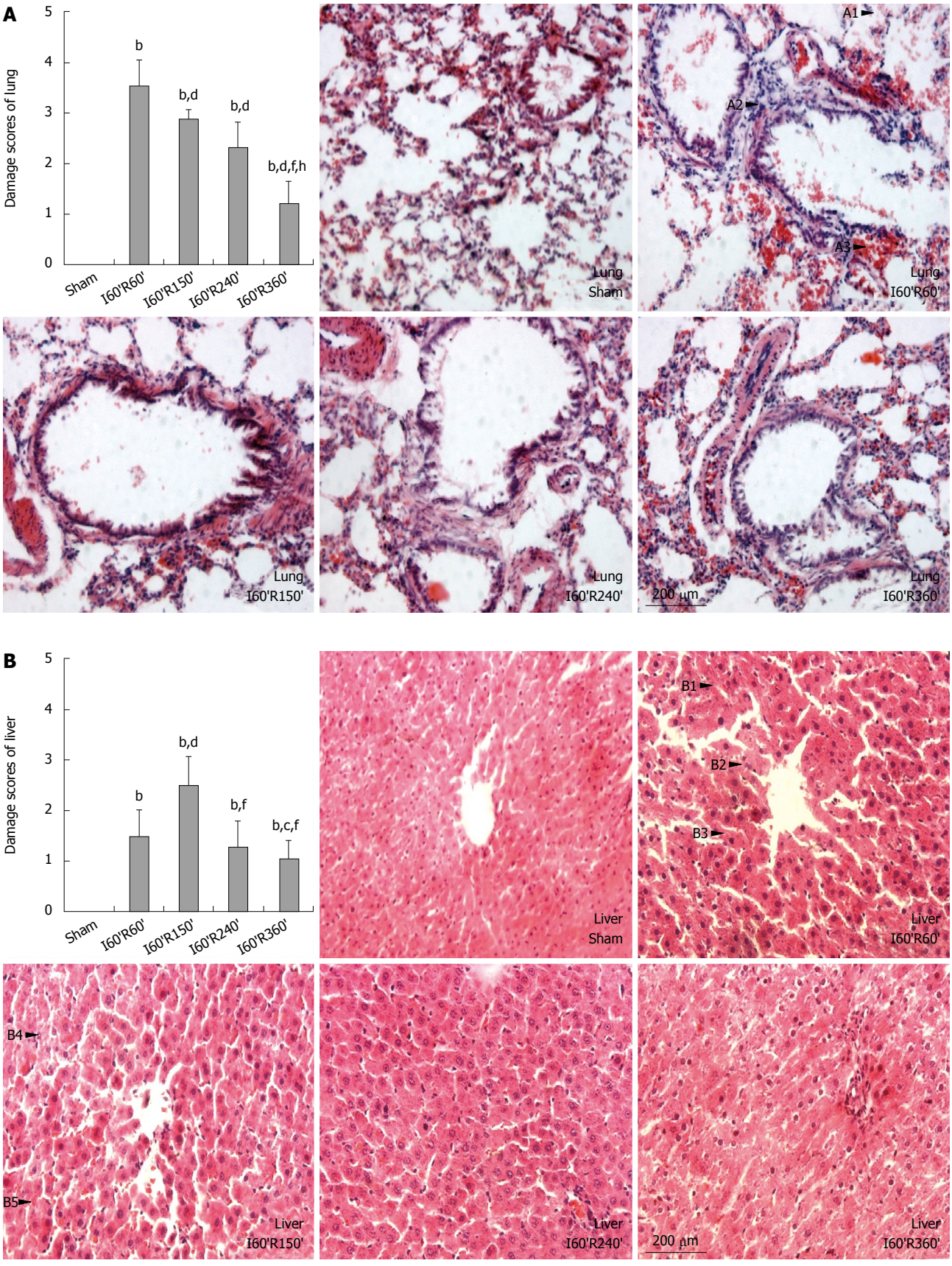
reperfusion groups was not different from that in Sham, but the level in I60'R360' was significantly lower than in I60'R60' (t = -2.411, $P < 0.05$), demonstrating a decreasing tendency as compared with that in I60'R150' (P = 0.086). No difference was observed in the levels among I60'R60', I60'R150' and I60'R240' (Figure 1F).

Histological alterations in lung, liver and duodenum after injury

Compared with pulmonary alterations in the Sham group after injury, alveolar walls in I60'R60' were disrupted (Figure 2, *A1*) and infiltrated by numerous inflammatory cells (*A2*), and the interstitial tissue was fulfilled with erythrocytes (*A3*). Similar but significantly alleviated alterations were observed in I60'R150' and I60'R240', while apparently minor disruption of alveolar structure, hypercellularity and vascular congestion became evident in I60'R360'. Damage scores of the lung in the four reperfusion groups were significantly higher than in the Sham (all $P < 0.01$), while the scores in I60'R150', I60'R240' and I60'R360' were significantly lower than in I60'R60' (z = -3.000, -2.810 and -2.762, all $P < 0.01$). The scores in I60'R150' and I60'R240' were significantly higher than in I60'R360' (z = 2.762 and 2.887, both $P < 0.01$), but without difference (Figure 2A).

Compared with hepatic alterations in the Sham group after injury, hepatic cords in I60'R60' were widened and arranged disorderly, hepatic sinusoids were narrowed or vanished (Figure 2, *B1*), hepatocytes were found swollen with loosened cytoplasm (*B2*) and a few Councilman bodies were scattered (*B3*). Apparently worsened vacuolization and lytic necrosis of hepatocytes (*B4*), increased Councilman bodies as well as vascular congestion (*B5*) were observed in I60'R150', while minor alterations similar with that in I60'R60' were found in I60'R240', and the lesions in I60'R360' were obviously attenuated. Damage scores of the liver in the four reperfusion groups were significantly higher than in the Sham (all $P < 0.01$), while the scores in I60'R60', I60'R240' and I60'R360' were significantly lower than in I60'R150' (z = -3.000, -2.810 and -2.739, all $P < 0.01$). The score in I60'R60' was significantly higher than in I60'R360' (z = 2.000, P = 0.0455), while no difference was found in the scores between I60'R240' and I60'R360', as shown in Figure 2B.

Compared with duodenal alterations in the Sham after injury, the ciliated epithelial cells in I60'R60' were impaired and arranged irregularly (Figure 2, *C1*), the quantity of Goblet cells (*C2*) and mucus secretion were increased, and mild congestion in the mucosal lamina propria was observed (*C3*). Distinctly aggravated impairment of the ciliated epithelial cells, further increased the quantity of Goblet cells but minor congestions were found in I60'R150' and I60'R240', while significantly alleviated altera-


A
Damage scores of lung
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I60'R240'
I60'R360'
Lung
Sham
A1
A2
A3
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I60'R60'
Lung
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Lung
I60'R240'
200 μm
Lung
I60'R360'
B
Damage scores of liver
5
4
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I60'R60'
I60'R150'
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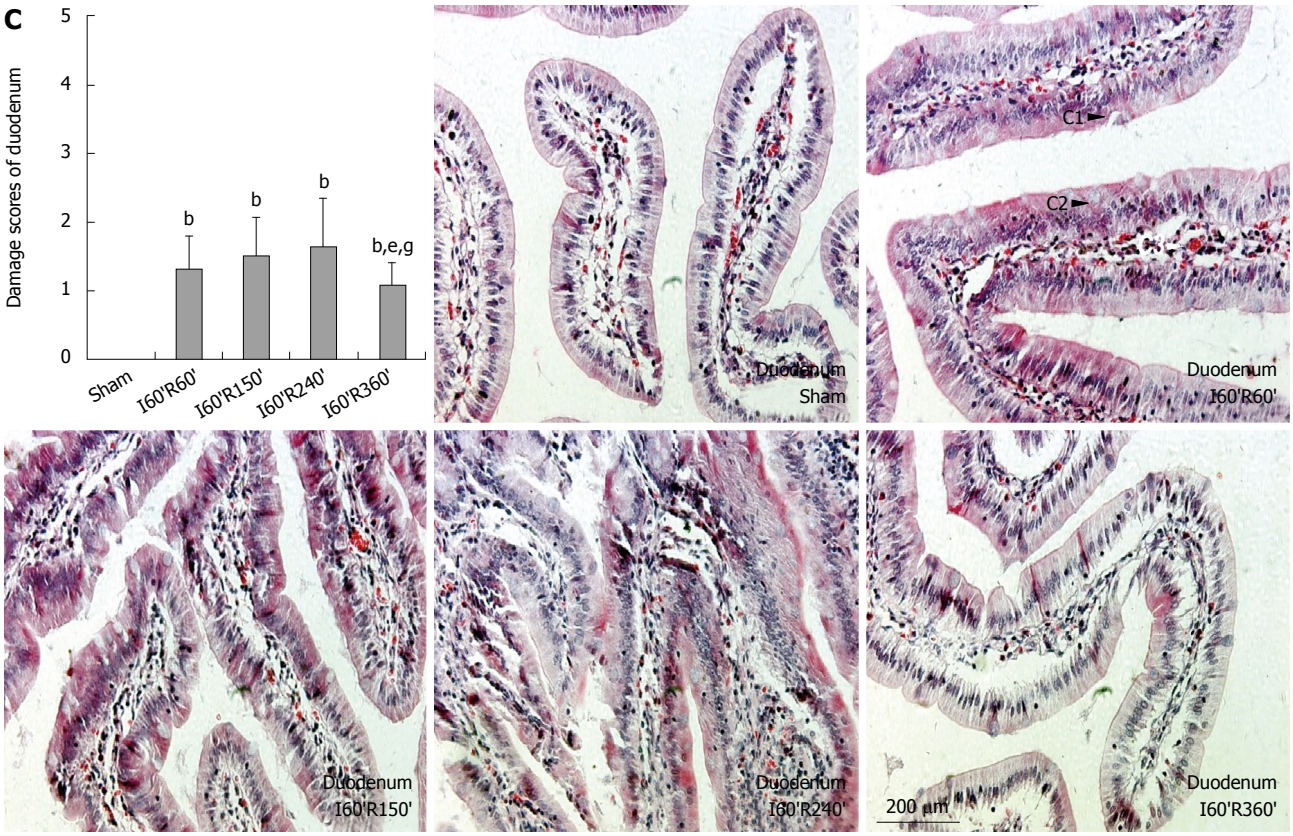


Figure 2 Histological alterations in lung (A), liver (B) and duodenum (C) of the rats after injury using hematoxylin-eosin staining (× 200). Sham: Sham-operation; I60'R60', I60'R150', I60'R240' and I60'R360': Hepatic ischemia for 60 min/reperfusion for 60, 150, 240 and 360 min; $^{b}P < 0.01$ *vs* sham; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* I60'R60'; $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* I60'R150'; $^{g}P < 0.05$, $^{h}P < 0.01$ *vs* I60'R240'.

tions were observed in I60'R360'. Damage scores of duodenum in the four reperfusion groups were significantly higher than in the Sham group (all $P < 0.01$), while the score in I60'R360' was significantly lower than in I60'R150' and I60'R240' ($z = -2.000$ and -1.987, both $P < 0.05$). No difference was found in the scores among I60'R60', I60'R150' and I60'R240' (Figure 2C).

Protein levels of leptin in lung, liver and duodenum after injury

In the lung, the protein levels of leptin in I60'R60', I60'R150', I60'R240' and I60'R360 were significantly lower than in the Sham ($t = -7.241$, -4.962, -11.970 and -10.821, all $P < 0.01$), while the level in I60'R150' was significantly higher than in I60'R60', I60'R240' and I60'R360' ($t = 4.633$, 13.096 and 11.086, all $P < 0.01$). The levels in I60'R240' and I60'R360' were significantly lower than in I60'R60' ($t = -5.166$ and -3.708, both $P < 0.01$), and the level in I60'R360' was significantly higher than in I60'R240' ($t = 3.046$, $P < 0.01$) (Figure 3A).

In the liver, the protein levels of leptin in I60'R60' and I60'R240' were significantly higher than in the Sham ($t = 13.769$ and 6.952, both $P < 0.01$), while the levels in I60'R150' and I60'R360' showed no difference as compared with that in the Sham. The levels in I60'R240' and I60'R360' were significantly lower than in I60'R60' ($t = -13.754$ and -4.683, both $P < 0.01$), while no difference was observed in the levels among I60'R150', I60'R240' and I60'R360', as shown in Figure 3B.

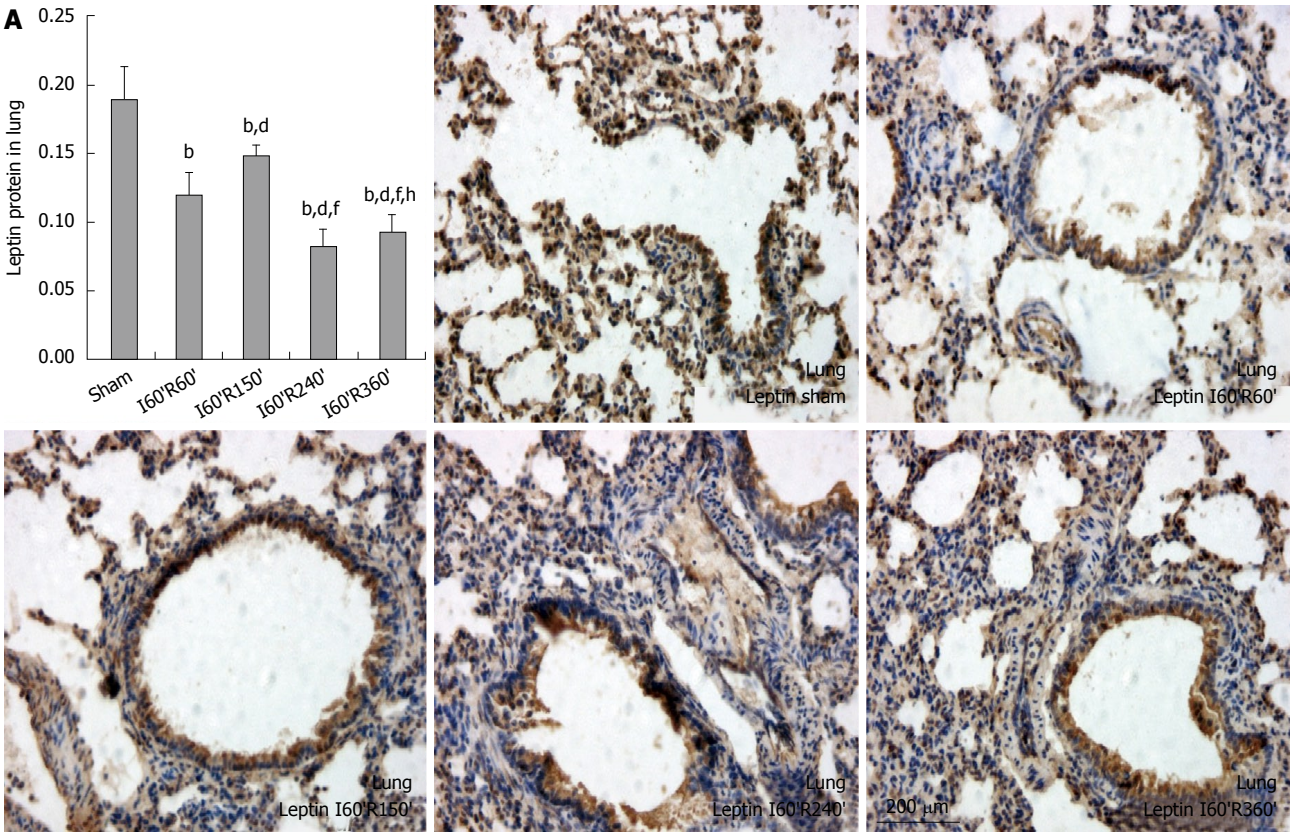
In the duodenum, the protein levels of leptin in I60'R240' and I60'R360' were significantly higher than in the Sham ($t = 22.847$ and 35.411, both $P < 0.01$), I60'R60' ($t = 6.890$ and 7.760, both $P < 0.01$) and I60'R150' ($t = 12.538$ and 14.237, both $P < 0.01$), while the levels in I60'R60' and I60'R150' were not different from that in the Sham. The level in I60'R150' was significantly lower than in I60'R60' ($t = -2.431$, $P < 0.05$), and there was no difference in the levels between I60'R240' and I60'R360' (Figure 3C).

Correlation between leptin and other variables

There was no significant correlation between serum leptin and glucose, TAC, MPO or DAO, while a significantly positive correlation was found between serum leptin and ALT ($\rho = 0.344$, $P = 0.021$).The protein level of leptin in the lung had significantly negative correlation with its damage scores ($\rho = -0.313$, $P = 0.036$), but the level in the liver showed a significantly positive correlation with its damage scores ($\rho = 0.297$, $P = 0.047$), and no significant correlation was observed between the levels in the duodenum and its damage scores (Table 1).

DISCUSSION

Despite advances in supportive therapy, hepatic ischemia/reperfusion injury continues to negatively affect patient mortality and morbidity[10]. As the liver is the most impor-


A
Leptin protein in lung
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I60'R150'
I60'R240'
I60'R360'
Lung
Leptin sham
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Leptin I60'R60'
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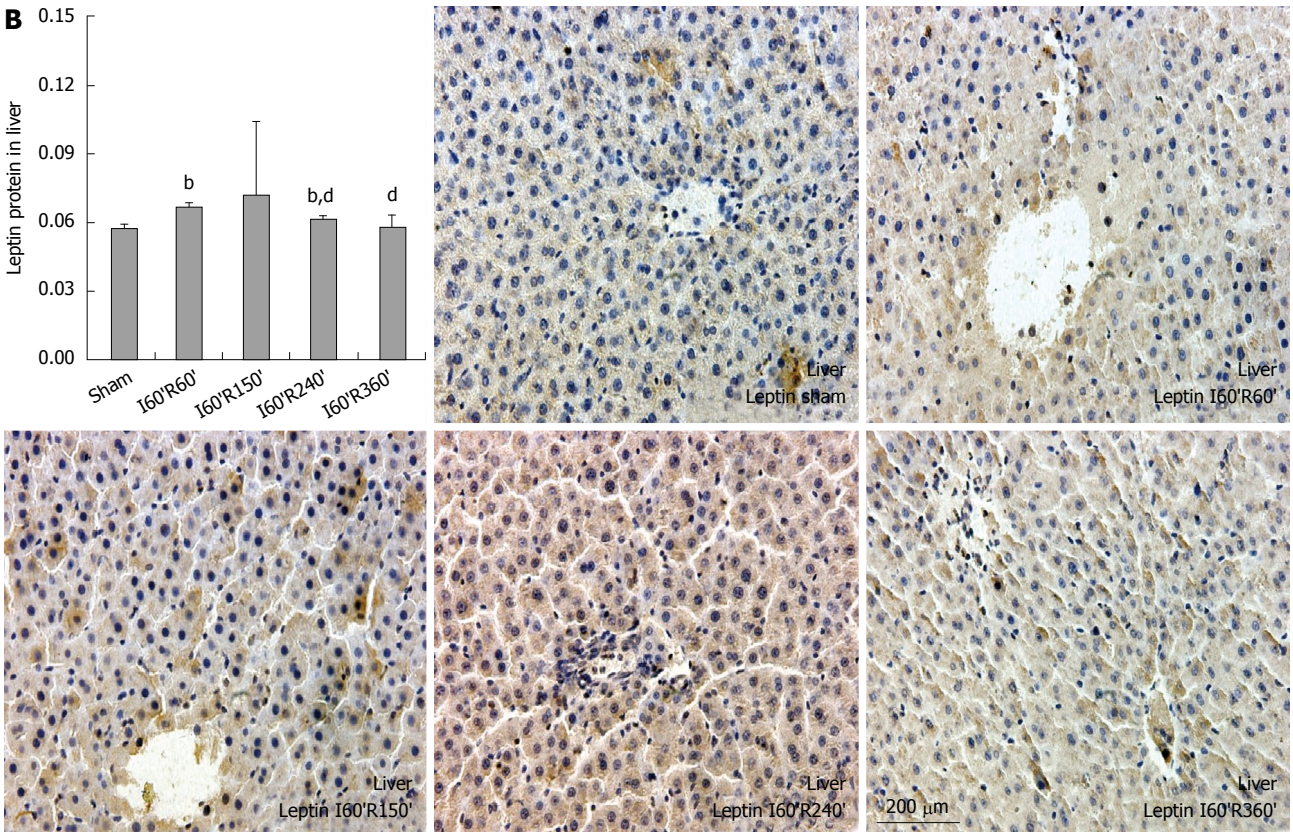
Lung
Leptin I60'R360'
200 μm


B
Leptin protein in liver
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Sham
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I60'R240'
I60'R360'
Liver
Leptin sham
Liver
Leptin I60'R60'
Liver
Leptin I60'R150'
Liver
Leptin I60'R240'
Liver
Leptin I60'R360'
200 μm

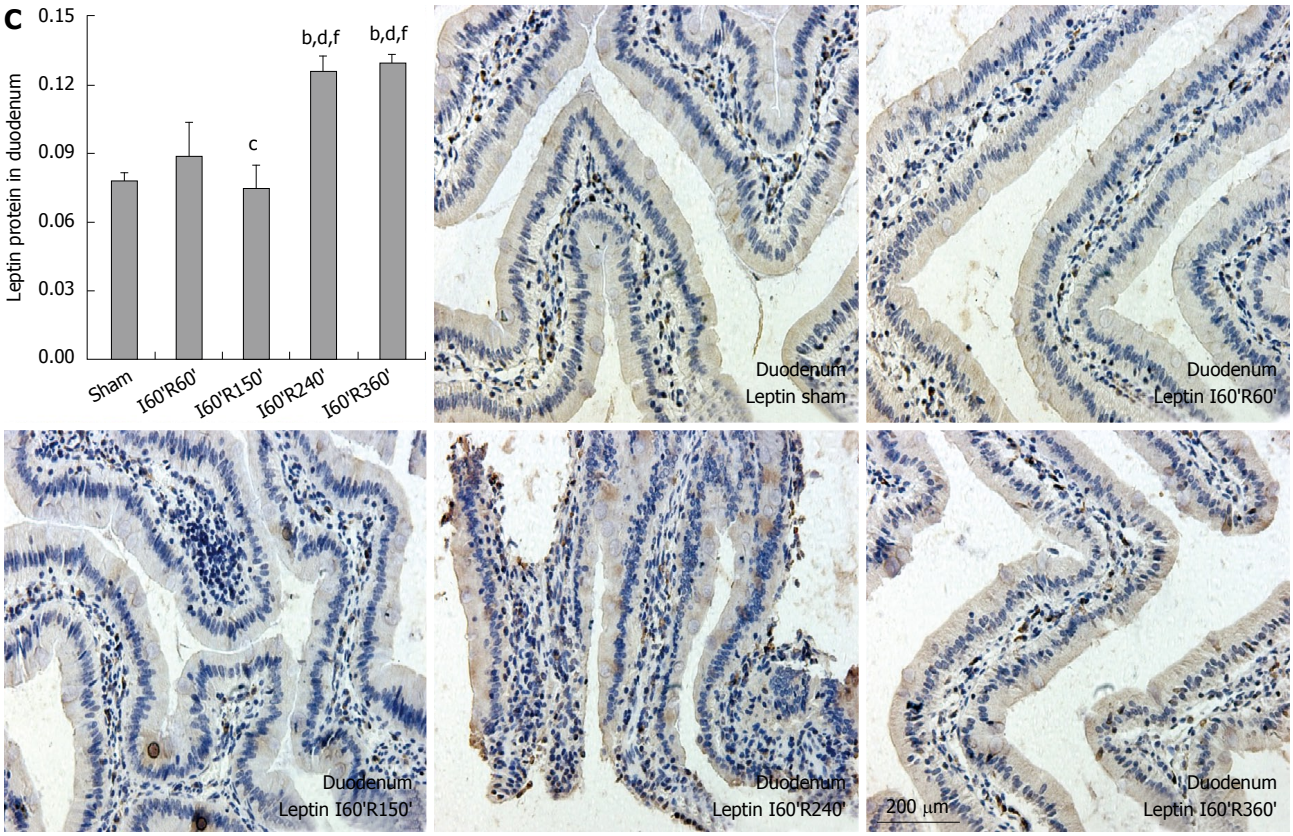

Figure 3 Protein levels of leptin in lung (A), liver (B) and duodenum (C) of the rats after injury detected by immunohistochemistry (× 200). Sham: Sham-operation; I60'R60', I60'R150', I60'R240' and I60'R360': hepatic ischemia for 60 min/reperfusion for 60, 150, 240 and 360 min; $^{b}P < 0.01$ *vs* sham; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* I60'R60'; $^{f}P < 0.01$ *vs* I60'R150'; $^{h}P < 0.01$ *vs* I60'R240'.

Table 1 Correlation analysis between leptin and other variables

Analyzed pairs	ρ	*P*
Serum leptin + glucose	-0.061	0.693
Serum leptin + total anti-oxidation capacity	0.254	0.092
Serum leptin + myeloperoxidase	-0.083	0.586
Serum leptin + alanine transaminase	0.344	0.021
Serum leptin + diamine oxidase	-0.098	0.523
Tissue leptin + damage scores of lung	-0.313	0.036
Tissue leptin + damage scores of liver	0.297	0.047
Tissue leptin + damage scores of duodenum	0.180	0.237

ρ and *P* values were calculated using Spearman's rank correlation.

tant organ participating in energy metabolism and nutrient absorption, this injury will inevitably lead to variation of endogenous metabolic factors such as leptin. Recent researches have suggested a protective role for leptin in trauma/sepsis-induced organ dysfunction[11,12], but there is no report mentioning the affirmatory linkage between leptin and hepatic ischemia/reperfusion injury, thus our study was the first to investigate whether leptin is a novel strategy aimed at minimizing the internal disorders after this injury. In the early stage after reperfusion, the high metabolic status changed the body into a starvation-like situation[13]. As leptin is down-regulated by the neuroendocrine system during starvation[14] and there exists a possible destructive effect of free radicals on circulating proteins, serum leptin from I60'R60' to I60'R240' remained significantly unchanged though their mean values seemed to increase. In the later stage, the starvation-like status and the negative effect of free radicals recovered gradually, therefore serum leptin in I60'R360' increased significantly and this elevation fluctuation indicated a potential benefit of leptin during this injury.

As serum glucose and TAC represent the energy reserves and the overall ability to eliminate reactive oxygen species, respectively, we chose them as variables reflecting metabolic disorders after hepatic ischemia/reperfusion injury. Although a decrease in serum glucose was observed during starvation[14], the stimulating effect by catecholamines release in the early stage after stress[15] reversed the descent, and no significant change of serum glucose was observed in I60'R60' to I60'R240'. As time went on, the starvation-like status and catecholamines release underwent synergic recovery, but serum glucose in I60'R360' was distinctly higher due to a possible feedback provocation of resumptive energy reserve. Hepatic ischemia/reperfusion injury is characterized by Kupffer cell-induced oxidant stress[16]; our results showed that the anti-oxidation system responded promptly and exerted a valid capacity to eliminate free radicals from I60'R240' to I60'R360', suggesting that a competent endogenous regulation might exist to accelerate the recovery of this injury. The synchronous increase of serum leptin, glucose and TAC in the later stage also indicated a potentiality of leptin to

protect the body by promoting energy metabolism and anti-oxidation capacity. Unexpectedly, no significant correlation was found either between serum leptin and glucose or between serum leptin and TAC, which might be attributed to the non-unique and complex regulation on glucose and TAC by other metabolic factors.

In liver transplantation, prolonged intensive care unit stay or lack of an adequate nutritional support results in starvation of the donor, leading to increased incidence of hepatocellular injury and primary dysfunction[17,18]. Given the fact that leptin decreases under malnutrition and starvation[14,19], it is attractive to speculate that leptin may associate with the liver impairment (even in other organs) during hepatic ischemia/reperfusion injury, and its elevation may proceed to maintain homeostasis. MPO is mainly expressed by neutrophils with a similar amount of nearly 5% of net weight in each cell[20], and it may indirectly reflect the severity of lung injury evoked by neutrophil infiltration. ALT and DAO are sensitive indicators for liver and duodenal injuries, thus we selected these three variables for representing the functional impairment in the lung, liver and duodenum (the distal, local and proximal vital organs), respectively. Our results showed that both serum MPO and DAO in the later stage after reperfusion were significantly lower than in the early stage, while serum ALT in the four reperfusion groups were higher than in Sham but were not aggravated as reperfusion time was elongated. These changes demonstrated that the functional impairment in the vital organs during 70% hepatic ischemia/reperfusion injury were relatively minor compared with 100% injury, and there should be an endogenous regulation to restrict deterioration of this injury and accelerate its recovery, which also indicated a potential benefit of leptin since it elevated synchronously in the later stage. Interestingly, no significant correlation was observed either between serum leptin and MPO or between serum leptin and DAO, while there was a significantly positive correlation between serum leptin and ALT, suggesting a direct protective role for leptin in the local vital organ but an indirect regulatory role in the distal and proximal vital organs during this injury.

A research has revealed that serum leptin is apparently higher in patients after liver, heart or kidney transplantation[21], which provides a clinical probability of leptin as a beneficial cytokine for ischemia/reperfusion-induced organ injuries. However, no histological alterations and their association with leptin were reported. Our results showed that although hepatic ischemia/reperfusion injury initiated distinct and diverse structural impairment, the severity in the later stage after reperfusion were alleviated compared with the early stage, suggesting once again that an endogenous regulation did exist to stimulate the recovery of this injury. Protein levels of leptin in the lung, liver and duodenum underwent different fluctuations due to disparate grades of structural impairment, with a timely response in the lung and liver in the early stage but a delayed response in the duodenum in the late stage, which also indicated a regulatory role for tissue leptin during the injury. Perplexingly, a significantly negative correlation between tissue leptin in the lung and its damage scores was observed, suggesting a conflicting role for leptin in the lung injury. Two critical researches have found that lung injury is moderately attenuated in leptin-deficient mice and intratracheal pretreatment with a leptin receptor inhibitor mitigates leptin-induced lung edema[22,23]. Our investigation also suggested that endogenous leptin might be detrimental to the recovery of lung injury despite that exogenous leptin has been proved beneficial for lung and other organs[24-26]. The significantly positive correlation between tissue leptin in the liver and its damage scores indicated once again a direct protective role for leptin in the local vital organ, while the indistinctive correlation between tissue leptin in duodenum and its damage scores, but a contrary variation in the later stage, still provided a rehabilitative potency of leptin in the proximal vital organ by inhibiting its structural impairment.

Although many studies have been conducted regarding the inflammatory mechanisms during hepatic ischemia/reperfusion injury[16,27,28], little is known about the endogenous network involving leptin that modulates the internal disorders in this injury. Ischemia/reperfusion-evoked activation of Kupffer cells results in production and release of reactive oxygen species and pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), which further activate and recruit neutrophils into target organs especially in the lung, and they contribute to the inflammatory cascade and have powerful effects on the local environment and remote organ functions[13,29]. Since a rise in TNF-α activity parallels an increase in leptin gene and peripheral expression, it is proposed that leptin participates in a protective mechanism against the inflammatory cascade downstream to TNF-α[30,31]. Despite a decrease in serum glucose and leptin observed in the starvation-like status which augmented susceptibility to inflammatory damage, the synchronous upregulation of serum leptin, glucose and TAC in the late stage still declared a potential rehabilitative role of leptin in metabolic disorders during this injury. Although an ambiguous induction by leptin in the structural impairment in the lung was presented, leptin directly inhibited the functional and structural impairment in the liver, while it potentially suppressed the functional impairment in the lung as well as the functional and structural impairment in the duodenum. Therefore, leptin evolves to harmonize an endogenous network maintaining homeostasis in the distal, local and proximal vital organs during this injury (Figure 4).

In our experimental model, different doses of exogenous leptin can be administered at different time points during the injury, and relevant inflammatory cytokines or growth factors can be measured to elucidate the rehabilitative characteristics of leptin. Signal pathway downstream to leptin can be investigated simultaneously to further clarify the association between leptin and the internal disorders. Moreover, liver cell lines may be used to establish a hypoxia/reoxygenation model, and applied to determine whether endogenous regulation or exogenous supplement of a functional leptin network will represent a beneficial component for the host response to the injury.

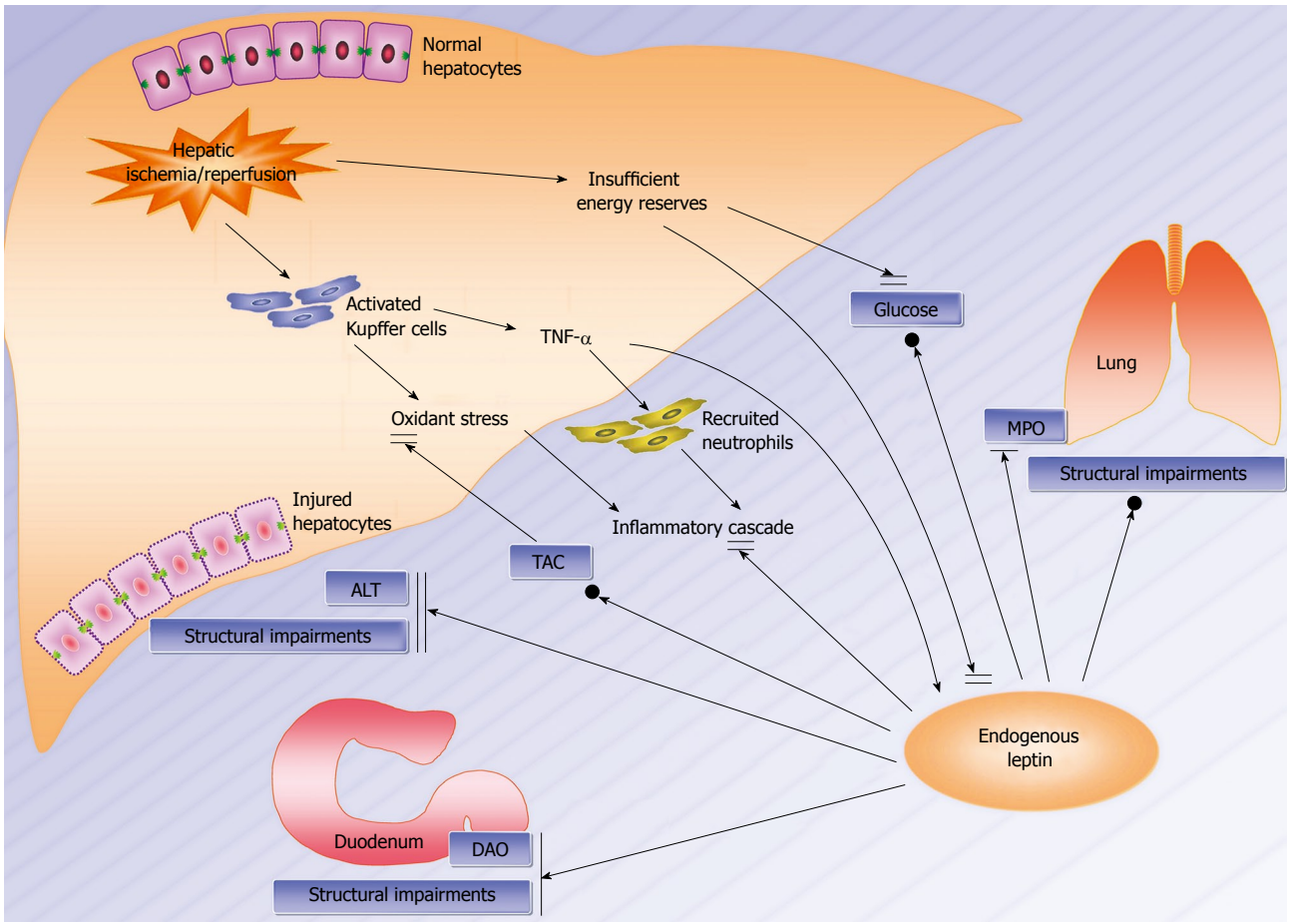

Figure 4 Endogenous network involving leptin that modulates the internal disorders during hepatic ischemia/reperfusion injury. →: Induction; →||: Inhibition; →●: Possible induction; →|: Possible inhibition; TNF: Tumor necrosis factor; TAC: Total anti-oxidation capacity; MPO: Myeloperoxidase; ALT: Alanine transaminase; DAO: Diamine oxidase.

In conclusion, endogenous leptin fluctuates in serum and vital organs during hepatic ischemia/reperfusion injury, and exerts a potency to rehabilitate metabolic disorders as well as the functional and structural impairment in the lung, liver and duodenum, especially in the local vital organ. The association between leptin and the internal disorders has suggested that an artificial nutritional support, adequate content of antioxidants and exogenous leptin recruitment aimed to upregulate endogenous leptin may represent a new supportive therapy for hepatic ischemia/reperfusion injury.

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COMMENTS

Background

Recent researches have revealed that leptin participates in the modulation of energy metabolism, neuroendocrine, angiogenesis, reproduction and immune responses, suggesting an important role of leptin in the recovery of functions. To study the fluctuation of leptin and its association with the internal disorders after hepatic ischemia/reperfusion injury may provide a potential target for the treatment of hepatic ischemia/reperfusion injury.

Research frontiers

Liver is the vital organ for nutrition absorption and energy metabolism, its ischemia/reperfusion injury inevitably leads to local and remote dysfunction. As leptin is a stress mediator proceeding to maintain homeostasis and there is no available report about leptin and this injury, to explore the variation and roles of leptin will clarify whether it is a beneficial component for this injury.

Innovations and breakthroughs

This is the first study to report that endogenous leptin fluctuates in serum and vital organs in a rat model of 70% hepatic ischemia/reperfusion injury, and exerts a potency to rehabilitate metabolic disorders, the functional and structural impairment in the lung, liver and duodenum, especially in the local vital organ.

Applications

This study suggests that endogenous leptin may represent a potential target for minimizing the internal disorders after hepatic ischemia/reperfusion injury, and an artificial nutritional support, adequate content of antioxidants and exogenous leptin recruitment aimed to upregulate endogenous leptin may provide a new supportive therapy for this injury and relevant diseases.

Terminology

Hepatic ischemia/reperfusion injury is a complication of liver resection, transplantation and hypovolemic shock, resulting in depletion of adenosine triphosphate, accumulation of free radicals, and lesion in vital organs. Leptin is an *ob* gene-expressed protein with a primary role of inhibiting food intake, modulating weight balance and promoting energy metabolism, but its potential role in this injury has not been reported.

Peer review

The authors illustrated a well designed experiment to evaluate the role for

leptin in the internal disorders during hepatic ischemia/reperfusion injury. Furthermore, relevant background information was given in order to interpret the importance of the trial. The manuscript is interesting and comprehensive. The part of discussion is compactly constructed and brilliant.

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BRIEF ARTICLE

Absence of high amplitude propagating contractions in subjects with chronic spinal cord injury

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Abstract

AIM: To investigate the presence or absence of high amplitude propagating contractions (HAPC), as well as the other measures of colonic motility, in persons with spinal cord injury (SCI).

METHODS: Prolonged colonic ambulatory manometric studies were performed on 14 male volunteers: 8 with SCI (mean age, 59 ± 13 years; mean duration of injury, 13 ± 4 years) and 6 healthy able-bodied controls (mean age, 57 ± 10 years). A solid-state manometry catheter was endoscopically clipped to the splenic flexure. Recording was performed for > 24 h after manometric catheter placement.

RESULTS: HAPC were absent in individuals with SCI during pre-sleep, sleep, and post-sleep phases. HAPC were significantly increased after awakening in non-SCI controls (0.8 ± 0.2 HAPC/h *vs* 10.5 ± 2.0 HAPC/h, $P < 0.005$). The motility index was lower in those with SCI than in controls pre- and post-sleep (SCI *vs* non-SCI: Pre-sleep, 2.4 ± 0.4 *vs* 8.8 ± 1.9, $P < 0.01$; Post-sleep, 4.3 ± 0.8 *vs* 16.5 ± 4.5, $P < 0.05$). However, a sleep-induced depression of colonic motility was observed in both the SCI and non-SCI groups (Pre-sleep *vs* Sleep, non-SCI: 8.8 ± 1.9 *vs* 2.1 ± 0.9, $P < 0.002$; SCI: 2.4 ± 0.4 *vs* 0.2 ± 0.03, $P < 0.001$), with the motility index of those with SCI during sleep not significantly different than that of the controls.

CONCLUSION: HAPC were not observed in individuals with SCI pre- or post-sleep. A sleep-induced depression in general colonic motility was evident in SCI and control subjects.

Key words: Spinal cord injury; Neurogenic bowel; Bowel motility; High amplitude propagating contractions; Motility index

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INTRODUCTION

Neurogenic bowel dysfunction (NBD) is a sequela of spinal cord injury (SCI) that leads to a disruption of normal colon physiology. This condition may be characterized by constipation, diarrhea, fecal incontinence, and/or fecal impaction, and it has a profoundly negative impact on quality of life[1]. In chronic SCI, colonic motor function is disturbed and often presents as difficulty with evacuation, associated with a delay in colonic transit, as evidenced by several colon marker transit studies[2-4]. In addition, our group has reported that colonic contractions after SCI are of lower amplitude and are decreased in frequency compared to those in healthy able-bodied controls[5].

High amplitude propagating contractions (HAPC) are specialized propagating pressure waves with high amplitude (> 105 mmHg) and prolonged duration (> 14 s) observed on manometric recordings[6]. HAPC are often associated with colonic mass movements and are thought to be a precursor of bowel evacuation; they occur mostly after arousal from sleep and after ingestion of meals[7,8]. In a study of non-SCI patients with chronic slow transit constipation, HAPC were either absent or attenuated after arousal from sleep and after ingestion of a meal, and such a finding may be indicative of neurogenic or myopathic colon dysfunction[9].

Given the above findings, it is possible that alterations in HAPC may play a role in the pathophysiology of bowel evacuation in persons with SCI. However, characteristics of HAPC and their relationship to normal physiological stimuli in persons with SCI have not been reported. Thus, our study determined HAPC in relation to sleep in individuals with chronic SCI.

MATERIALS AND METHODS

Subjects

Fourteen male volunteers were studied: 8 with chronic SCI and 6 non-SCI controls. The mean age of the patients with SCI was 59 ± 13 years with a mean duration of injury of 13 ± 4 years. Three of the 8 SCI subjects had tetraplegia (C5 or below) and the remaining 5 had paraplegia (T5 or below). The causes for SCI included trauma (6/8), cervical stenosis (1/8) and transverse myelitis (1/8). Persons with SCI who reported having < 2 spontaneous bowel movements per week were recruited for the study; they had participated in a regular bowel care program for at least 6 mo prior to enrollment in the study. None of the SCI patients enrolled in the study had autonomic dysreflexia. The non-SCI subjects had no previous history of gastrointestinal disease or surgery, had normal physical examinations, and had no electrolyte or thyroid abnormalities. Six healthy non-SCI individuals, aged 57 ± 10 years, served as the control group. Medications that may alter colonic motility and/or hemostasis (e.g. warfarin, aspirin, *etc.*) were withheld for 1 wk prior to the study. The Institutional Review Board of the James J Peters Veterans Affairs Medical Center approved the study protocol, and informed consent was obtained from subjects before enrollment.

Sleep phases

The pre-sleep phase was designated as the time (1 h) before the start of sleep. The reported start and end of sleep was quantified in hours and was designated as the sleep phase. The post-sleep phase was designated as the time (1 h) after the end of sleep. The information regarding sleep duration was obtained from nursing records and self-reported diaries. Subjects avoided strenuous physical activity for at least 1 h before or after sleep.

Manometry assembly and experimental design

All subjects had normal colonic examinations by colonoscopy before the study. Subjects were prescribed either polyethylene glycol (PEG) (4 L of Colyte) or oral sodium phospho soda (OSPS) (two divided doses of Fleets PhosphoSoda 45 mL each separated by 12 h). Sedation with propofol was administered when needed. Fixation of the manometric probe to the wall of the colon at the splenic flexure was accomplished, as previously described[10]. A solid state manometric catheter with four pressure transducers spaced 10 cm apart was used for obtaining intraluminal pressure data (Gaeltec Ltd, Dunvegan, Isle of Skye, UK). The colonoscope and the manometric catheter probe were advanced under direct vision to the splenic flexure. Using fluoroscopy, the last pressure sensor was placed at least 10 cm proximal to the anal verge. The distal end of the manometric probe was securely taped to the skin of the gluteal region to prevent accidental retraction; an abdominal X-ray confirmed the placement of the clipped probe at the region of the splenic flexure. An abdominal X-ray confirmed that all subjects had normal radiographic findings (i.e. none had megacolon). The manometric probe was attached to a portable recorder (type 7-MPR, Gaeltec Ltd., UK). The portable recorder was connected to a shoulder sling, permitting mobility. The subjects were provided with diaries and were instructed to record the timing of specific activities such as meals, sleep, and bowel and bladder events. Subjects were instructed that after a bowel movement care should be taken during perineal hygiene to prevent the inadvertent external displacement of the probe.

After completion of the study, the data were uploaded to a computer for storage and analysis. The probe remained securely in place after placement in the colon in all

subjects. Subjects remained in the hospital overnight for the duration of the study. Because of the possibility that bowel preparation, colonic intubation, and/or sedation may have effects on colonic motility, data collection was begun 12 h after placement of the manometry catheter. Non-SCI subjects meticulously documented events of walking, strenuous activity, movement, cough, sleep and wake, bladder emptying, periods of food ingestion and talking. Because activities may alter HAPC, tracings that corresponded to periods of increased activity were excluded from the data analysis.

From the manometric recordings of the colon, three epochs were determined a priori to measure and analyze the endpoints: pre-sleep phase (i.e. 1 h before sleep), sleep phase (i.e. entire sleep duration), and post-sleep phase (i.e. 1 h after arousal from sleep). The following quantitative and qualitative endpoints were investigated in each epoch: (1) the presence and number of HAPC; and (2) other colonic motility parameters. HAPC were defined as pressure wave sequences that: (a) migrated aborad; (b) travelled across three or more consecutive channels; (c) had an amplitude of > 105 mmHg; and (d) had a duration > 14 s[9]. Other colonic motility parameters measured were the motility index (MI) (i.e. the product of the mean amplitude and percent activity) and the total number of waves. Sleep duration and periods of food ingestion were obtained from nursing records for subjects admitted to the SCI Service and from self-report diaries for non-SCI subjects.

The recordings were analyzed by observing the manometric tracings on a computer monitor and by a software analysis program (AMBB, Gaeltec Ltd, UK). Criteria for waves included in the analysis were: (1) amplitude of > 8 mmHg and; and (2) duration of > 3 s[6]. Movement artifacts (e.g. walking, transferring, coughing, *etc.*) were confirmed by reference to the subject's diary, and these segments (as well as postprandial segments) were excluded from the analysis. The analyses were performed independently by investigators blinded to the subject's identity, and any discrepancies were resolved by the senior author, who was also blinded.

All subjects tolerated the procedure well. The probe was removed after the study by gently tugging along the catheter. No complications were noted after probe removal. For the analysis of HAPC pressure wave forms, data were excluded due to technical reasons in two individuals with SCI and in two healthy controls; in these cases, malfunction of more than one sensor for a period longer than 30 min prevented the ability to observe propagation across channels, hence, preventing the identification or quantification of HAPC.

Statistical analysis

The data were expressed as mean ± SE. All the data represent values obtained per hour. The mean values of the SCI and the control groups were analyzed for differences using unpaired Student's *t* test for parametric variables and Kruskal-Wallis Test for non-parametric variables. The means between phases of sleep were analyzed using paired Student's *t* test in both SCI and control groups. An analysis of variance (ANOVA) was performed to determine significance among the phases of sleep and arousal (i.e. pre-sleep, sleep, and arousal). Multiple regression analysis was performed to determine the effect of level of SCI on quantitative and qualitative wave parameters.

RESULTS

Subjects

In individuals with SCI, the level of injury did not have a significant effect on the quantitative and qualitative wave parameters. Thus, the data obtained in subjects with all levels of SCI were combined for further analysis. A total of approximately 420 h of manometric recording was obtained (range of 26-32 h per patient); 112 h were analyzed for the study. The average number of hours of sleep per subject was 8.0 ± 0.2 h (range: 7 to 10 h), without a significant difference in the average number of hours of sleep between SCI and non-SCI subjects. None of the subjects reported sleep interruption.

High amplitude peristaltic contractions

There were no HAPC observed in the SCI group during pre-sleep, sleep, and post-sleep phases. In the non-SCI group, the numbers of HAPC/h were as follows: pre-sleep phase, 2.0 ± 0.4; sleep phase, 0.8 ± 0.2; and post-sleep phase, 10.5 ± 2.0; a significant increase in the number of HAPC were noted from sleep to post-sleep phases (0.8 ± 0.2 *vs* 10.5 ± 2.0, $P < 0.005$). All non-SCI subjects experienced subjective symptoms (e.g. sensation of flatus) and two had bowel movements in close proximity to having HAPC in the post-sleep phase.

Motility index

In both the non-SCI and SCI groups, the colonic motility index (MI) was significantly suppressed during sleep compared to pre-sleep (Pre-sleep *vs* Sleep, Control: 8.8 ± 1.9 *vs* 2.1 ± 0.9, $P < 0.005$; SCI: 2.4 ± 0.4 *vs* 0.2 ± 0.03, $P < 0.001$) and significantly increased after arousal from sleep (Sleep *vs* Post-sleep, non-SCI: 2.1 ± 0.9 *vs* 16.5 ± 4.5, $P < 0.001$; SCI: 0.2 ± 0.03 *vs* 4.3 ± 0.8, $P < 0.001$) (Figure 1).

Furthermore, the MI during the pre-sleep and post-sleep phases was significantly lower in SCI subjects than that of the non-SCI group (SCI *vs* non-SCI: Pre-sleep, 2.4 ± 0.4 *vs* 8.8 ±1.9, $P < 0.01$; Post-sleep, 4.3 ± 0.8 *vs* 16.5 ± 4.5, $P < 0.05$). However, no significant difference in MI was noted during the sleep phase between SCI and non-SCI groups (SCI *vs* non-SCI: Sleep, 0.2 ± 0.03 *vs* 2.1 ± 0.9, $P = 0.10$).

Total number of waves

In both the control and SCI groups, the total number of waves was significantly suppressed during sleep compared to pre-sleep (Pre-sleep *vs* Sleep, non-SCI: 98 ± 11 *vs* 70 ± 5, $P < 0.005$; SCI: 68 ± 11 *vs* 51 ± 2, $P < 0.001$). The total number of waves was significantly increased in both groups after arousal from sleep (Sleep *vs* Arousal, non-

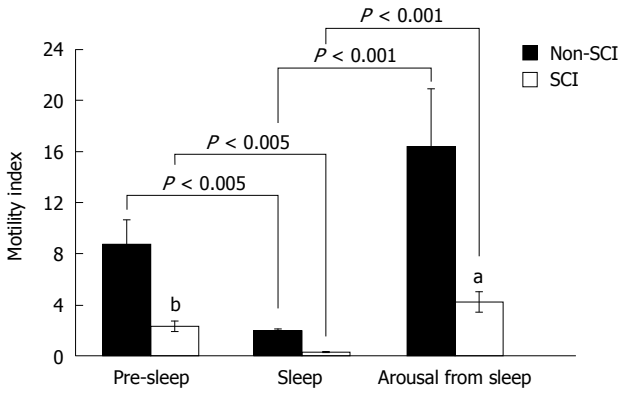

Figure 1 Colonic motility index in relation to sleep. In both the non-spinal cord injury (SCI) and SCI groups, colonic motility index (MI) was significantly suppressed during the sleep phase compared to the pre-sleep phase (Pre-sleep *vs* Sleep, Control: 8.8 ± 1.9 *vs* 2.1 ± 0.9, $P < 0.005$; SCI: 2.4 ± 0.4 *vs* 0.2 ± 0.05, $P < 0.001$) and significantly increased after awakening from sleep (Sleep *vs* Arousal, Control: 2.1 ± 0.9 *vs* 16.5 ± 4.5, $P < 0.001$; SCI: 0.2 ± 0.03 *vs* 4.3 ± 0.8, $P < 0.001$). The MI during the pre-sleep and post-sleep phases was significantly lower in SCI subjects than in the non-SCI (non-SCI *vs* SCI, pre-sleep: 8.8 ±1.9 *vs* 2.4 ± 0.4, $^{b}P < 0.01$; post-sleep: 16.5 ± 4.5 *vs* 4.3 ± 0.8, $^{a}P < 0.05$).

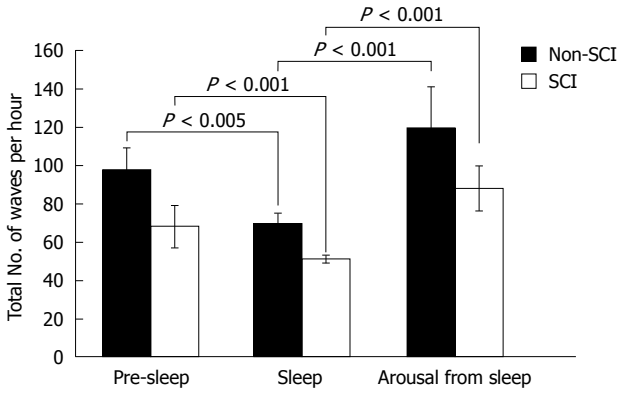

Figure 2 Total number of waves in relation to sleep. In both the non-spinal cord injury (SCI) and SCI groups, the total number of waves was significantly suppressed during the sleep phase compared to the pre-sleep phase (Pre-sleep *vs* Sleep, non-SCI: 98 ± 11 *vs* 70 ± 5, $P < 0.005$; SCI: 68 ± 11 *vs* 51 ± 2, $P < 0.001$), and was significantly increased after awakening from sleep (Sleep *vs* Arousal, non-SCI: 70 ± 5 *vs* 120 ± 21, $P < 0.001$; SCI: 51 ± 2 *vs* 88 ± 12, $P < 0.001$).

SCI: 70 ± 5 *vs* 120 ± 21, $P < 0.001$; SCI: 51 ± 2 *vs* 88 ± 12, $P < 0.001$) (Figure 2).

DISCUSSION

As was previously reported in other studies[9,11-15], HAPC are often observed after awakening or occur after ingestion of a high fat meal[12]. Previously, our group studied the effects of food on colonic motility in persons with SCI and reported that the postprandial colonic response in persons with SCI is suboptimal and confined only to the descending colon[5]. In this study, we restricted our analysis to HAPC in relation to the sleep-wake cycle in subjects with SCI compared to those in able-bodied subjects. Our study revealed that HAPC are absent in individuals with chronic SCI who had reported having fewer than 2 spontaneous bowel movements per week. The absence of HAPC in persons with SCI may be related to abnormalities in the brain-gut control over colonic motility and/or intrinsic disorder of the viscus as a result of chronic SCI. The complete absence of the HAPC after chronic SCI may be a contributing factor to difficulty with evacuation. Our findings are consistent with the study of Rao *et al*[9] who demonstrated that absence of HAPC may indicate colonic neuropathy.

Interestingly, in both SCI and non-SCI subjects, there appeared to be a sleep-induced depression of colonic motor waves measured, i.e. motility index and total number of all waves. Furthermore, in both SCI and non-SCI subjects, there appeared to be a significant increase of peristaltic waves measured after awakening, except for HAPC.

HAPC are thought to be the manometric equivalent of colonic mass movements[16], and they are likely to be associated with transport of intraluminal contents along colonic segments[17]. Prolonged periods of colonic recording in man have reported HAPC to occur between 4 to 10 times per 24-h period[9,11,18]. In non-SCI subjects, 60% of HAPC were associated with symptoms or events (e.g. bowel movement or passage of gas)[1]. The interest in the role of HAPC as a variable that permits discrimination between states of health and disease is evolving, as more sophisticated methodology and technology become available to allow more accurate and prolonged colonic recording under physiological conditions[6,10,11,19,20]. More recently, in a study of patients with slow-transit constipation[9], HAPC were employed to categorize patients with slow-transit constipation as having colonic neuropathy based on the absence of two of three manometric criteria, one being HAPC and the other two being lack of gastrocolonic response and lack of response to waking. The patients who were categorized as having colonic neuropathy were considered to have a refractory colonic neuromuscular disorder; after further evaluation of gastric and small bowel motility, 7 of the 10 patients were advised to have, and subsequently underwent, surgery (i.e. colectomy with ileorectal anastomosis)[9].

The current study has limitations. Initially, the frequency and number of HAPC in the non-SCI group reported in this study were significantly higher than has been previously reported[15,18,19]. The reason for this disparity is not clear but could be due to technical differences, the small number of subjects and/or the study design. Regardless of the cause, the relative differences between SCI subjects and non-SCI controls remain significant because the same procedures and manometric sensors were employed in both groups. Furthermore, studies have shown that in addition to awakening from sleep, movement (e.g. ambulation) also precipitates the generation of HAPC. Thus, activity may have been a significant factor that may serve to explain the difference in HAPC generation in the SCI compared to the non-SCI group. We have carefully excluded motion artifact, especially those related to walking in the able-bodied subjects during the analysis of our wave recordings. However, mobility as a generator of HAPC *per se* could not be addressed because of our study cohort

(non-SCI) and our study design (e.g. non-SCI subjects were not confined to the seated position during the acquisition of data). The number of subjects studied was relatively small, and the SCI group was not uniform. Another limitation of this work was that the sleep phases were not confirmed by polysomnography; because of this limitation, we were also unable to determine if subjects had periods of arousal during the periods of reported sleep. In our study protocol, persons with SCI who reported having > 2 spontaneous bowel movements per week were excluded from study participation. Thus, another limitation of the study was that it did not address possible differences in HAPC in relation to sleep in the subjects with SCI with more normal bowel evacuation patterns (i.e. > 2 bowel movements per week) compared to those of the non-SCI subjects.

In conclusion, subjects with SCI had a complete absence of HAPC. The MI before and after sleep was depressed in persons with SCI compared to able-bodied subjects. Thus, SCI results in disruption of the colonic motility changes that are present in healthy individuals. In our subjects with SCI, a marked depression in sleep-induced colonic activity was demonstrated by the MI and the total number of waves, and this depression in activity related to sleep has also been confirmed, as expected, in healthy controls. The absence of HAPC generation is presumably an important factor in the occurrence of difficulty with evacuation observed in individuals with SCI. Prokinetic drugs, such as bisacodyl and neostigmine[21,22], that result in generation of HAPC, may improve bowel evacuation by increasing HAPC after SCI.

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BRIEF ARTICLE

Cortical and spinal evoked potential response to electrical stimulation in human rectum

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Abstract

AIM: To study a novel technique to record spinal and cortical evoked potentials (EPs) simultaneously in response to electrical stimulation in the human rectum.

METHODS: Eight male and nine female healthy volunteers participated. Stimulating electrodes were attached to the rectal mucosa at 15 cm and 12 cm above the dentate line. Recording skin electrodes were positioned over vertebrae L4 through S2. The electrical stimulus was a square wave of 0.2 ms duration and the intensity of the stimulus varied between 0 and 100 mA. EP responses were recorded using a Nicolet Viking IV programmable signal conditioner.

RESULTS: Simultaneous recording of cortical and spinal EPs was obtained in > 80% of the trials. The EP responses increased with the intensity of the electrical stimulation, were reproducible overtime, and were blocked by application of Lidocaine jelly at the site of stimulation. The morphology (N1/P1), mean ± SD for latency (spinal N1, 4.6 ± 0.4 ms; P1, 6.8 ± 0.5 ms; cortical N1, 136.1 ± 4.2 ms; P1, 233.6 ± 12.8 ms) and amplitude (N1/P1, spinal, 38 ± 7 μV; cortical 19 ± 3 μV) data for the EP responses were consistent with those in the published literature. Reliable and reproducible EP recordings were obtained with the attachment of the electrodes to the rectal mucosa at predetermined locations between 16 and 8 cm above the anal verge, and the distance between the attachment sites of the electrodes (the optimal distance being approximately 3.0 cm between the two electrodes).

CONCLUSION: This technique can be used to assess potential abnormalities in primary afferent neural pathways innervating the rectum in several neurodegenerative and functional pain disorders.

Key words: Spinal evoked potentials; Cortical evoked potentials; Rectum; Electrical stimulation; Primary afferent neurons; Visceral sensation

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INTRODUCTION

Evoked potential (EP) recordings in response to peripheral nerve stimulation are useful measures of the conduction characteristics of afferent sensory pathways. EP responses at the cortical level have been recorded in response to mechanical (balloon) or electrical stimulation in the gastrointestinal tract at several locations including the esophagus, colon and rectum[1-4]. These responses are reproducible, at least with electrical stimulation in the rectum[5,6]. The cortical EP responses reflect the cumulative involvement of peripheral and central afferent pathways. Recording spinal EPs that measure the conduction characteristics of the first order primary afferent neurons has been challenging because of technical difficulties. For example, recording spinal EPs in response to balloon distention in the rectum is problematic because the stimulation artifact associated with balloon distension overlaps with the ability to record spinal waveforms with latencies typically < 20 ms[7]. The availability of a reliable method to record visceral EPs at the spinal level would help address an important unresolved issue that involves several gastrointestinal disorders in which dysfunction of primary afferent neurons has been implicated. For instance, visceral hypersensitivity has been reported in patients with irritable bowel syndrome but it remains unresolved whether this disorder is associated with distinct abnormalities involving the first order primary afferent neurons that transmit pain signals and/or higher order processing in pain signaling pathways[8]. We could identify only one publication that has reported simultaneous recording of cortical and spinal EPs in response to visceral stimulation[7]. In that study, recording of cortical EPs in response to rectal electrical stimulation was observed in approximately 90% of subjects, whereas spinal EPs were recorded simultaneously with cerebral EPs in approximately 40% of the subjects (personal communication). This supports the need for improved methods to record spinal EPs in response to stimulation of visceral primary afferent neurons.

Here, we report a new technique to record spinal and cortical EPs simultaneously in response to electrical stimulation in the rectum, which increased the percentage of successful spinal EP recordings to approximately 80%. We propose that the reliability of this method was related to the direct attachment of stimulating electrodes to the rectal mucosa *via* rigid sigmoidoscopy at specific locations, and the distance between the sites of electrode attachment.

MATERIALS AND METHODS

Seventeen healthy male (n = 8) and female (n = 9) subjects, with a mean age of 32 years (range: 20-44 years), completed the recording session. None of the volunteers had a history of medical, gastrointestinal, or neurological problems, or were taking any medication on a regular basis. The protocol was approved by the Institutional Review Board at the University Michigan Medical Center. Written informed consent was obtained from all participants.

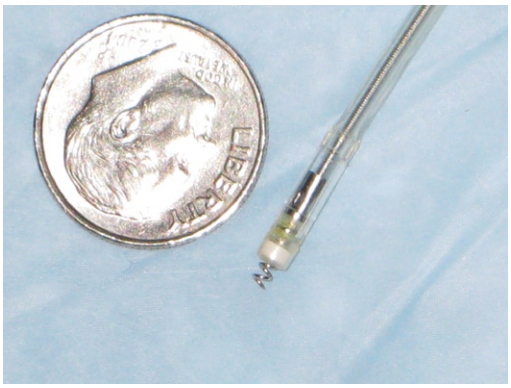

Figure 1 Stimulating electrodes. Two electrodes (Medtronic CapSureFix Model 5076) were used in this study. This model had an electrically active helix that was designed to extend easily (up to 2 mm) for active fixation and retraction. The electrodes were positioned at 15 cm and 12 cm above the anal verge.

Rectal electrical stimulation protocol

Subjects fasted overnight before the study to eliminate the confounding influence of recent food consumption. A 200-mL tap water enema was administered prior to the recording session to ensure adequate visualization of the rectum. The participant was positioned in the left lateral decubitus position. All studies were performed in a quiet room with dimmed lighting. Two electrodes (Medtronic CapSureFix Model 5076, unipolar configuration) were used in this study (Medtronic Inc., Minneapolis, MN, USA) (Figure 1). This model has an electrically active helix that is designed to extend easily (up to 2 mm) for active fixation and retraction. The electrodes were attached to the rectal mucosa *via* a disposable rigid sigmoidoscope (Welch Allyn, Model 53130; All Medical Supply, Westland, MI). Typically, placement of the stimulating electrodes was accomplished in 2-3 min with minimal discomfort to the participant.

In preliminary studies, we examined placement of the electrodes at varying distances above the dentate line from 6 to 20 cm, and varied the proximal-distal distance between the attachment sites of the two electrodes from 0.5 to 4 cm. Preliminary studies revealed that the optimal placement to record cortical and spinal EPs simultaneously was between 8 and 16 cm above the dentate line, and 2.5-3.5 cm separation between the two electrodes. Recording of spinal EPs was progressively unreliable at distances < 2.0 cm between the electrode attachment sites. Separation distances > 3.0 cm between the stimulating electrodes required progressively higher intensities of electrical stimulus to produce a threshold response. Based on the preliminary studies, we routinely attached the stimulating electrodes at 15 and 12 cm above the dentate line on the anterior wall of the rectum. A ground electrode was positioned at the back of the neck. Cortical and spinal EPs were recorded simultaneously using Ag/AgCl-skin-electrodes positioned at the vertex [midway between the auricular prominences (Cz)] referenced to linked ears (AlA2) and L4 and L5 and S1 and S2. In some studies, skin electrodes were also attached at L1-3 to provide ad-

ditional spinal EP data. EP responses were recorded using a Nicolet Viking IV programmable signal conditioner (Nicolet Biomedical, Madison, WI, USA). The electrode impedances were < 5 kΩ. Each trial represented the final average of 50 randomly applied stimulations (every 1-3 s) that was repeated three times to ensure intra-run reproducibility. The random stimulation paradigm was employed to minimize any potential contribution of stimulus anticipation by the participant.

The electrical stimulus was a square wave of 0.2 ms duration and the intensity of the stimulus varied between 0 and 100 mA. Recording parameters: sample frequency 2 kHz, amplifier gain 100 K, bandpass filters at 0.5-500 Hz for cortical EPs and 30-10 kHz for spinal EPs. EP waveforms exhibited characteristic prominent negative (N) and positive (P) peaks numbered in order of occurrence, i.e. N1, P1, *etc.* All amplitude and latency measures were calculated from the responses obtained at the Cz and L5 locations because in most subjects, the waveforms were best defined and highest in amplitude at these locations. Relative positivity resulted in a downward deflection in the waveform. The latency (ms) of the spinal and cortical responses was measured at the peak of the major negative deflection (N1) and the positive peak (P1), respectively. The spinal and cortical response amplitude (μV) was measured peak-to-peak (N1/P1). The latency reflects the conduction velocity characteristics of the afferent pathway being investigated and, in the absence of temporal dispersion, the amplitude of a signal reflects the number of afferents contributing to the response[5,6].

Volunteers were instructed before stimulation to report when they first perceived any sensation in the pelvic area. Electrical pulses were initiated at a current shown to be sub-threshold (< 5 mA) and gradually increased by 1-mA increments until perception was reported. All subjects reported a threshold non-painful pulse deep in the pelvis. This was identified as the threshold intensity. The paradigm was repeated three times to ensure reproducibility of the threshold sensation and associated EP profile, and again at current intensities of 1.5 and 2 times above the threshold intensity. The threshold for sensation always corresponded to the initial appearance of the EP waveform. It is noteworthy that in two recording sessions, the EP responses became erratic. In both cases, one of the stimulating electrodes had detached from the mucosa. Reattachment of the electrode to the mucosa resulted in restoration of a reproducible cortical and spinal EP response.

After documenting that the EP waveform was reproducible by repeating each trial three times, the stimulating electrodes were removed in six participants and the rectal mucosa at the site of attachment was swabbed with Lidocaine (Xylocaine) 2% topical jelly (AstraZeneca, Wilmington, DE, USA), using a cotton applicator under direct visualization using the rigid sigmoidoscope. The stimulating electrodes were then reattached at their original sites and the response to electrical stimulation repeated using the same parameters (1.5 times threshold intensity) that were employed to produce the initial EP signal. In addition, six participants underwent repeat testing between 1 and 6 mo after the first recording session, to evaluate reproducibility of the initial EP responses.

Table 1 Summary amplitude data for graded electrical stimulation (mean ± SD)

N1/P1 (μV)	Spinal	Cortical
Threshold	21 ± 4	12 ± 3
2.0 × threshold	49 ± 6	26 ± 5

Table 2 Summary data for latencies and amplitudes for spinal and cortical evoked potentials (mean ± SD)

	Spinal	Cortical
N1 latencies (ms)	4.6 ± 0.4	136.1 ± 4.2
P1 latencies (ms)	6.8 ± 0.5	233.6 ± 12.8
N1/P1 amplitude (μV) (1.5 × threshold)	38 ± 7	19 ± 3

Statistical analysis

For acquisition and analysis of spinal and cortical EP recordings, the stimulus intensity used was 50% (1.5 ×) greater than threshold stimulus. Results were compared by two-tailed Student's *t* test for paired observations. Repeated measures analysis of variance was used to compare the latency and amplitude data from the two recording sessions. The level of significance for all calculations was set at the 95% confidence level ($P < 0.05$).

RESULTS

Reproducible polyphasic cerebral EPs were recorded in 16 of the 17 subjects (94%) who participated in the study. Simultaneous spinal and cortical EPs were recorded in 14 of the 17 participants (82%). All participants demonstrated the typical morphology for cortical and spinal EP recordings of an N1/P1 wave form depicted in Figure 2, which increased with stimulus intensity (threshold intensity, left panel; 2 × threshold intensity, right panel). Note the different scales for measuring the amplitude of EP response in the left panel (cortical, 5 μV/division; spinal, 20 μV/division) and right panel (cortical, 10 μV/division; spinal, 50 μV/division). Summary amplitude data for graded electrical stimulation (threshold and 2 × threshold) are presented in Table 1. The mean threshold stimulus intensity was 28 mA (range: 12-47 mA), which was experienced as a non-painful pulse deep within the pelvis; typically in the midline or left of midline. Participants used the descriptors of a "tapping" or "poking" sensation that increased in intensity and corresponded to the intensity of electrical stimulation. The mean ± SD values for latency and amplitude of the EP responses obtained at a stimulus intensity of 1.5 × threshold for the 14 participants who demonstrated both cortical and spinal EP responses are presented in Table 2.

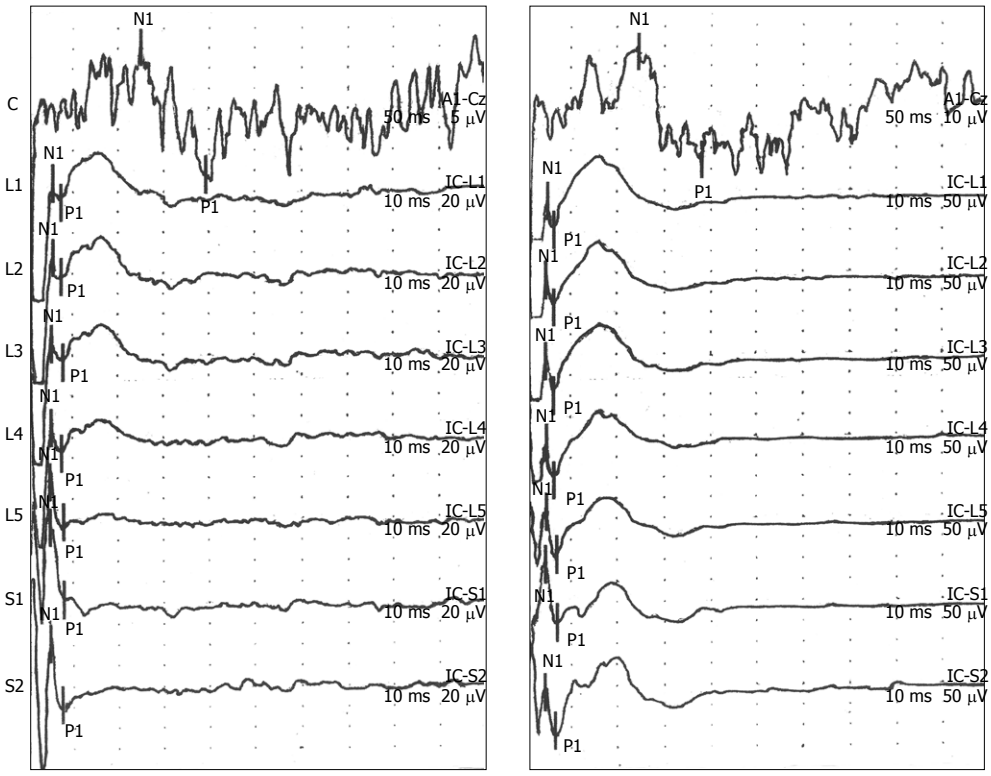

Figure 2 Representative cortical and spinal evoked potential responses to electrical stimulation in the rectum. Evoked potential (EP) responses demonstrated that the typical morphology for cortical and spinal EP recordings including an N1/P1 wave form that increased with stimulus intensity (threshold intensity, left panel; 2 × threshold intensity, right panel). Note the different scales for measuring the amplitude of EP response in the left panel (cortical, 5 μV/division; spinal, 20 μV/division) and right panel (cortical, 10 μV/division; spinal, 50 μV/division). Summary latency and amplitude results for recordings obtained using an electrical stimulus 1.5 × threshold is presented in Table 2.

Table 3 Summary data for reproducibility of latencies and amplitudes for spinal and cortical evoked potentials with repeat testing (mean ± SD)

	Spinal	Cortical
N1 latencies (ms)	4.4 ± 0.5	132.1 ± 6.1
P1 latencies (ms)	6.6 ± 0.6	229.8 ± 15.8
N1/P1 amplitude (μV)	35 ± 8.5	18.6 ± 3.4

The six participants who underwent a second recording session demonstrated reproducible EP wave forms and no significant changes in the latency and amplitude values compared to the first recording session (Table 3). Representative EP responses from two recording sessions for one volunteer are presented in Figure 3.

Application of Lidocaine jelly to the rectal mucosa at the sites of initial stimulation followed by reattachment of the electrodes at the original locations, and restimulation using the same parameters resulted in loss of the EP wave forms at the spine and vertex. In addition, all participants experienced a loss in the perception of the tapping or poking sensation in the pelvis after application of Lidocaine (Figure 4, right panel, $n = 6$).

DISCUSSION

The goal of this study was to evaluate a novel approach to stimulate the rectal mucosa electrically and simultaneously record EPs at the level of the lower spinal column and vertex. We were able to record successfully simultaneous cortical and spinal EPs in approximately 80% of the volunteers, which represents a twofold improvement over a previous study[7]. We were unable to record reliable spinal EPs in three of 17 participants. In these three individuals, we were able to record a cortical EP waveform. We speculate that the inability to record a reproducible spinal wave form in these three individuals may be related to body habitus. All three individuals had a body mass index > 25.

The polyphasic cortical EP wave forms that we recorded demonstrated similar morphology, latencies and amplitudes as described in previous studies[1-4]. We believe that any differences compared to the published literature can be attributed to the methodology that we employed, which involved direct attachment of the electrodes to the rectal mucosa, and the distance between attachment of the electrodes. The sensory innervation of the rectum involves afferent fibers that travel with sympathetic and parasympathetic spinal afferent nerves. The pelvic nerve afferents originate from the inferior hypogastric plexus[9] include a mixture of unmyelinated C fibers and poorly myelinated Aδ fibers that transmit a variety of sensory modalities that range from innocuous to painful[10-12]. Stimulation in this area is complicated by the proximity of

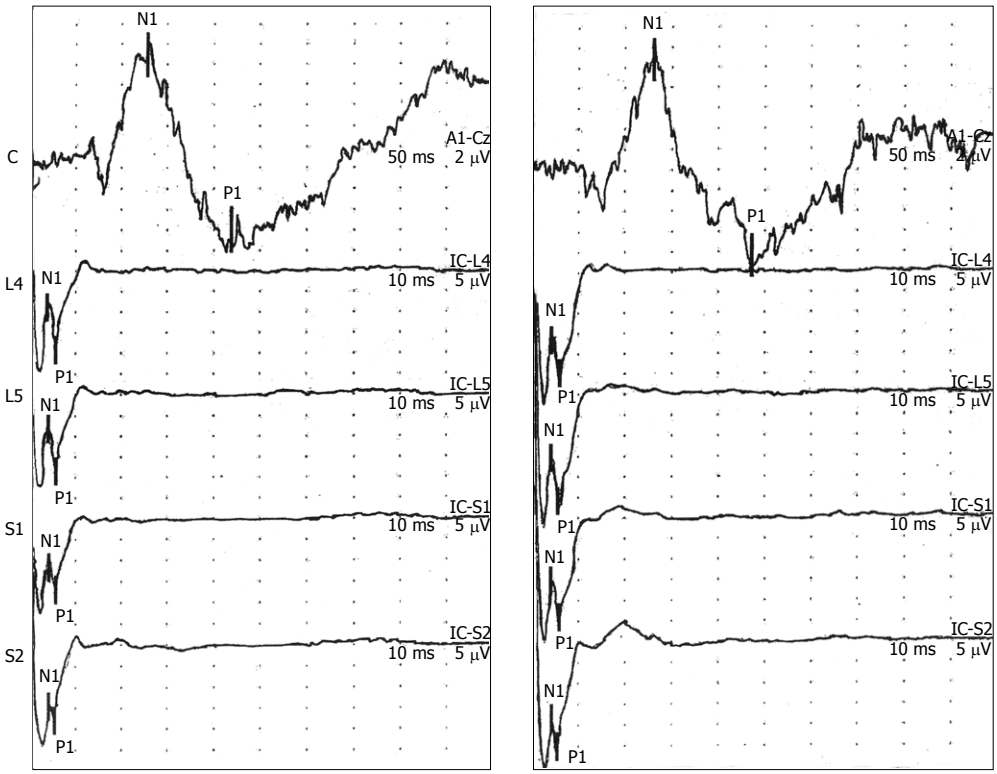

Figure 3 Evoked potential responses were reproducible over time. Representative cortical and spinal evoked potential responses from two recording sessions for one volunteer are shown in this figure. No significant changes in the latency and amplitude values were observed compared to the initial recording session. Summary data are presented in Table 3.

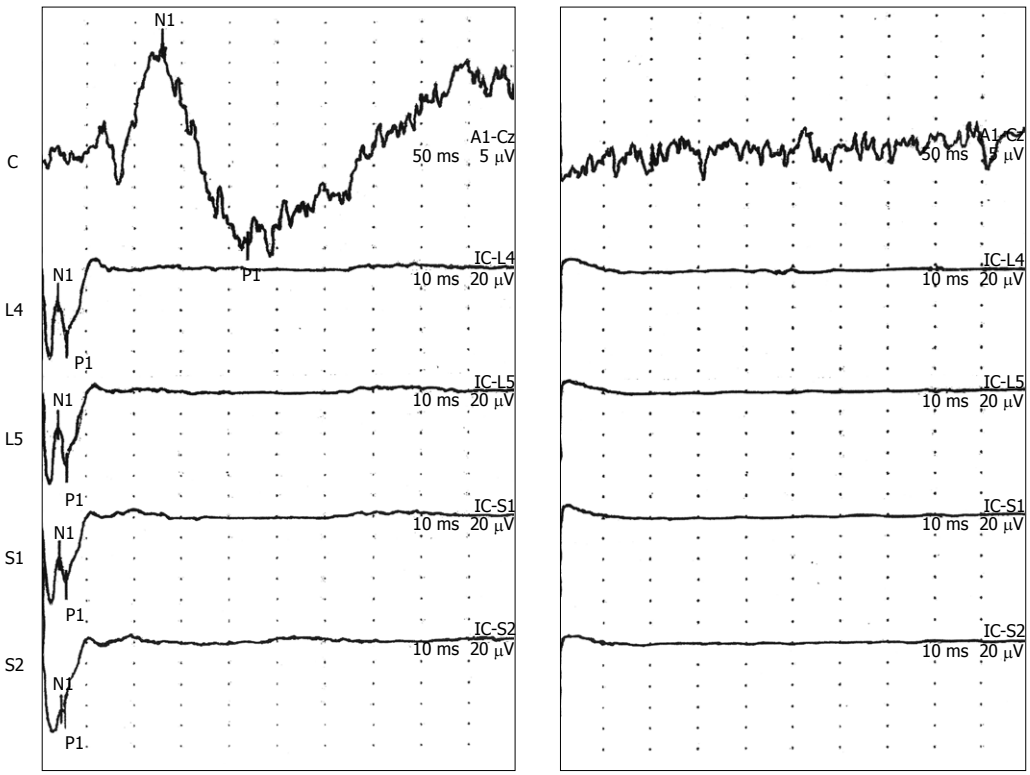

Figure 4 Application of Lidocaine jelly to the rectal mucosa at the sites of electrical stimulation resulted in loss of the cortical and spinal evoked potential responses. A representative evoked potential response (before and after application of Lidocaine) is presented in this figure. In addition, all participants reported a loss in the sensory response to electrical stimulation after application of Lidocaine.

somatic nerves that could be activated by high-intensity electrical stimulation.

Very few published data exist with regard to the characteristic wave form, latencies and amplitudes for spinal EPs using rectal stimulation. The characteristics of the spinal EP wave forms that we recorded were quite similar to those in a previous study that used a different electrical stimulation methodology[7]. Neurophysiological testing has been employed in the diagnoses of neurogenic disorders that involve the lower urinary tract and spinal cord, including somatosensory EP measurements[13,14]. However, the performance of cortical and spinal EP testing in response to bladder or urethra stimulation has not gained broad acceptance in clinical practice because of technical challenges.

We employed electrical stimulation in this study because currently available technologies using balloon distention obscure the recording of spinal EP wave forms. A potential drawback of using electrical stimulation is that this approach can result in nonspecific activation of all afferent pathways, depending on the stimulation parameters. On the other hand, it is possible that specific electrical stimulation parameters can be used to stimulate preferentially subpopulations of primary afferent neurons. For example, using our stimulation parameters, participants reported the perception of a non-painful "tapping" or "poking" sensation that increased with the intensity of electrical stimulus. This is similar to previous studies[1,3,4]. Others have used electrical stimulation protocols (typically involving high stimulation intensities) that evoke the perception of pain[8]. The sensations reported by our participants may be mediated by Aδ-fiber somatic afferents that are thought to encode non-painful stimuli, but saturate at stimulation levels well below pain threshold. It is possible that customized electrical stimulus parameters might prove useful to assess Aδ-fiber afferents in isolation. This may be important in sensitization states because this population of afferents can undergo phenotypic changes after the development of central sensitization[12]. Pain perception is thought to involve slower conducting small myelinated Aδ and unmyelinated C fibers and require more intense stimulation for activation[15]. It remains to be determined whether individuals with neurodegenerative disorders or chronic pain syndromes will demonstrate detectable changes in latency or amplitude of EP wave forms recorded at the lower spinal.

In conclusion, this study demonstrates that is possible to record cortical and spinal EPs simultaneously using rectal electrical stimulation with a relatively high level of reliability, e.g. approximately 80%. We believe that this methodology can be employed to improve our understanding of the potential contribution of rectal primary afferent neurons in various neurodegenerative and functional pain disorders. We anticipate that combining this technology with other assessments of anorectal physiology and functional neuroimaging techniques will result in novel insights regarding the neurophysiological characteristics of visceral afferent pathways in both health and disease.

COMMENTS

Background

Recording of evoked potentials (EPs) is a broadly applied technique to study central and peripheral neural function in health and disease in humans. Recording of EPs at the level of the spine in response to visceral stimulation has been technically challenging. The absence of a reliable method to record spinal EPs in response to visceral stimulation has placed limitations on the study of primary afferent neurons and their role in a variety of neurodegenerative and functional pain disorders. This article reports a new technique that represents a significant improvement in our ability to record cortical and spinal EPs simultaneously in response to electrical stimulation in the human rectum.

Research frontiers

At the present time, we have a limited understanding of the role of the primary sensory neurons that innervate the lower gastrointestinal tract in neurodegenerative diseases such as diabetes mellitus or functional pain disorders such as irritable bowel syndrome. The availability of a technique to record spinal EPs reliably in response to visceral stimulation will help address this deficit in our knowledge base.

Innovations and breakthroughs

The authors believe that the improved reliability of this method to record spinal EPs in response to visceral stimulation is related to the attachment of the stimulating electrodes to the rectal mucosa at specific locations, and the distance between the stimulating electrodes.

Applications

The authors believe that this methodology can be employed to improve their understanding of the potential contribution of rectal primary afferent neurons in various neurodegenerative and functional pain disorders. The authors anticipate that combining this technology with other assessments of anorectal physiology and functional neuroimaging techniques will result in novel insights regarding the neurophysiological characteristics of visceral afferent pathways in both health and disease.

Terminology

An EP is an electrical potential recorded from the nervous system of a human or other animal following presentation of a stimulus such as sound (auditory EP), light (visual EP), or physiological [chemical, mechanical, heat/cold or electrical (somatosensory EP)]. They can be contrasted to spontaneous potentials detected by electroencephalography or electromyography.

Peer review

The authors of the present study report a new technique to measure spinal and cortical EPs simultaneously in response to visceral stimulation. This technique could enable us to determine whether alterations in visceral hypersensitivity involve abnormalities in the first-order primary afferent neurons.

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BRIEF ARTICLE

Entecavir as treatment for reactivation of hepatitis B in immunosuppressed patients

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Abstract

AIM: To study the efficacy and safety of entecavir (ETV) as first-line therapy for hepatitis B virus (HBV) reactivation due to immunosuppression.

METHODS: Four patients that were treated with different immunosuppressive regimens for hematological malignancies, who presented with HBV reactivation were treated with ETV. Clinical outcome, biochemical and virological factors, including quantitative hepatitis B surface antigen (HBsAg) were studied.

RESULTS: In all patients, ETV induced suppression of HBV, and rapid clinical improvement without side effects. In one patient with an alanine aminotransferase (ALT) flare, tenofovir was added after 3 mo of treatment. Until death from disease progression at 6 mo after treatment initiation, this patient did not clear HBV infection. Retrospectively, it is highly probable that the patient had been non-adherent. In the other three patients, the virological responses were associated with an expeditious decrease in quantitative HBsAg titers with negativity after 2 mo, and all three had HBsAg seroconversion. In one patient, HBV DNA reached a plateau after 3 mo, before becoming undetectable after 1 year, despite early ALT normalization and undetectable quantitative HBsAg.

CONCLUSION: ETV seems to be effective and safe treatment for HBV reactivation. Monitoring of quantitative HBsAg might be an additional useful tool to monitor treatment response.

Key words: Hepatitis B virus; Entecavir; Immunosuppression; Hepatitis B surface antigen; Seroconversion

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INTRODUCTION

In patients with previous exposure to hepatitis B virus (HBV), who are hepatitis B surface antigen (HBsAg)-positive, or HBsAg-negative and hepatitis B core antibody (anti-HBc)-positive, acute exacerbation may occur when they become immunocompromised[1]. HBV reactivation (HBV-R) can be severe and is associated with high fatality rates of up to 20%-30%. The European Association for

the Study of the Liver (EASL) now recommends that all patients who are planned to receive chemotherapy, immunosuppressive therapy or stem cell transplantation undergo a test for HBV serology, including anti-HBc antibody prior to treatment[2]. Prophylactic treatment is recommended for patients with positive HBsAg. For those with positive anti-HBc antibodies and undetectable HBV DNA, monitoring of serum alanine aminotransferase (ALT) and HBV DNA is recommended. Although this consensus for the prevention of HBV-R is now implemented in current guidelines, an effective management strategy for patients who are experiencing HBV-R has not yet been established.

Lamivudine is the most commonly used drug for HBV-R. It has been shown to be safe in reactivation and in severe acute or fulminant hepatitis B[3]. However, prognosis remains poor despite lamivudine therapy if hepatic decompensation occurs[4,5]. Furthermore, the efficacy of lamivudine is hampered by the high rate of drug resistance mutations within the HBV polymerase gene that are associated with treatment failure. Entecavir (ETV) was approved in 2006 for the treatment of chronic hepatitis B and offers the advantage of a higher resistance barrier than lamivudine. The EASL guidelines note that the use of tenofovir or ETV might be considered in HBV-R with high viral load, although there is very limited experience.

The aim of our study was to assess the efficacy and safety of ETV in patients with immunosuppression-associated HBV-R at our center.

MATERIALS AND METHODS

We report on four patients with hematological malignancies, who had been referred to our hepatology department because of HBV-R, after approval of ETV, in August 2006. Median age of the patients was 63 years (Table 1). Patient 1 was male, and the other three were female. Two patients had received hematopoietic stem cell transplantation (HSCT) for treatment of acute myeloid leukemia (AML) (patients 1 and 3), the other two patients suffered from follicular lymphoma (patient 2) and B-cell chronic lymphocytic leukemia (B-CLL) (patient 4, Table 1). The patient with CLL was treated with bendamustine. The patient with follicular lymphoma was managed by observation without specific anticancer treatment, but had been treated intermittently with methylprednisolone for rheumatoid arthritis and was thus, due to hematological malignancy and steroid treatment, considered immunocompromised at the time of HBV-R.

In all patient, HBV DNA was measured prospectively with real-time PCR (Abbott HBV rtPCR; Abbott, Wiesbaden, Germany). On-treatment serum HBsAg kinetics were analyzed retrospectively (Abbott HBSAG). HBsAg was assessed at baseline, during treatment (1-3 mo), and during follow-up.

Only one of the patients (patient 3) had HBV screening prior to immunosuppression and was diagnosed with resolved hepatitis B with surface antigen clearance and an anti-hepatitis B surface antigen antibody (anti-HBs) level of 370 IU/L (Abbott AUSAB). During chemotherapy, quantitative HBsAg titers and HBV DNA were monitored closely and remained negative. When anti-HBs level fell below 10 IU/L, lamivudine prophylaxis was initiated and continued until 4 mo after cessation of immunosuppression with cyclosporine. At this time, HBV DNA was not detectable. HBV-R occurred 8 mo after lamivudine treatment had been stopped.

Prior to the initiation of chemotherapy or immunosuppression, all patients had normal liver values. None of them had a history of prior non-viral liver disease. Co-infection with hepatitis A, C or delta viruses or HIV was excluded serologically at the time of referral to our unit.

RESULTS

At the time of diagnosis of HBV-R, all patients presented with similar clinical, laboratory and virological findings. All patients suffered from fatigue, nausea and jaundice. They all had moderate to high HBV-DNA levels that ranged from 1×10^5 to 8.06×10^7 IU/mL. ALT levels were > 10 times the upper limit of normal in all patients. In one patient, serum bilirubin concentration was normal. In the other three patients, bilirubin levels ranged from 8 to 20 mg/dL. International normalized ratio (INR) was within the normal range in three patients. Only one patient had an impaired liver function with an INR of 1.4 (patient 1). Three of the patients were hepatitis B e antigen (HBeAg)-positive (Table 1).

When HBV-R was diagnosed, all patients were immediately treated with ETV. Three treatment-naïve patients were treated with oral 0.5 mg/d ETV (Baraclude®; Bristol-Myers-Squibb, Munich, Germany), and one patient (patient 3) received 1 mg ETV because of prior prophylactic treatment with lamivudine.

In all patients, ETV induced suppression of HBV that was associated with rapid clinical improvement and ALT normalization (Figure 1A). Three of the four patients had undetectable HBV-DNA levels after 3, 5 and 13 mo of treatment, respectively (Figure 1B).

One of the patients (patient 4) died because of disease progression of CLL after 6 mo of treatment with ETV. After 3 mo of ETV treatment, tenofovir was added because chemotherapy had to be restarted and HBV DNA showed a 4-$\log_{10}$ reduction but was still detectable at about 10^4 IU/mL, and ALT flared. When the patient died after 6 mo of ETV treatment and 3 mo of add-on tenofovir treatment, ALT had normalized and HBV DNA had declined to 136 IU/mL. The patient was tested for ETV resistance by sequencing the s-gene and pol-gene, however, no resistance-associated mutations were detected. Further investigation revealed that this patient had been non-adherent and treatment had been suspended for 8 d because she had run out of medication.

Three patients had been HBeAg-positive and all of them had HBeAg seroconversion during the course of treatment. Three of four patients (patients 1-3) even had HBsAg loss after 1, 2 and 4 mo of treatment, respectively.

On-treatment quantitative serum HBsAg levels decreased rapidly and were undetectable at 8 wk in all pa-

Table 1 Baseline characteristics and clinical outcome of patients treated with entecavir for hepatitis B virus reactivation

	Patient No.			
Parameter	**1**	**2**	**3**	**4**
Age (yr)	66	57	51	78
Sex	M	F	F	F
Disease entity	AML	FL	AML	B-CLL
HBV serology prior to chemotherapy	ND	ND	anti-HBc pos., anti-HBs pos., HBs-Ag neg.	ND
Chemotherapy/treatment	HSCT	Prednisone	HSCT	Bendamustine
Peak ALT (IU/L)	870	3290	2592	711
Peak bilirubin level (mg/dL)	20	10	4.1	1
INR	1.4	1.0	1.0	1.0
Time of clinical disease presentation	9 mo after HSCT	During prednisone treatment	27 mo after HSCT; 8 mo after cessation of LAM prophylaxis	During current chemotherapy
HBV-DNA (IU/mL) at time of HBV reaction	8.06×10^7	5.65×10^5	1.00×10^5	4.50×10^7
HBeAg at time of reactivation	Positive	Negative	Positive	Positive
HBeAg loss	Yes	NA	Yes	Yes
HBsAg loss	Yes	Yes	Yes	No
Withdrawal of antiviral treatment	No	Yes	Yes	No
Anti-HBs (IU/L)	42	361	277	-
Clinical outcome	Alive	Alive	Alive	Malignancy related death

AML: Acute myeloid leukemia; B-CLL: B-cell chronic lymphocytic leukemia; FL: Follicular lymphoma; LAM: Lamivudine; ND: Not determined; HBV: Hepatitis B virus; HSCT: Hematopoietic stem cell transplantation; NA: Not applicable; INR: International normalized ratio; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase.

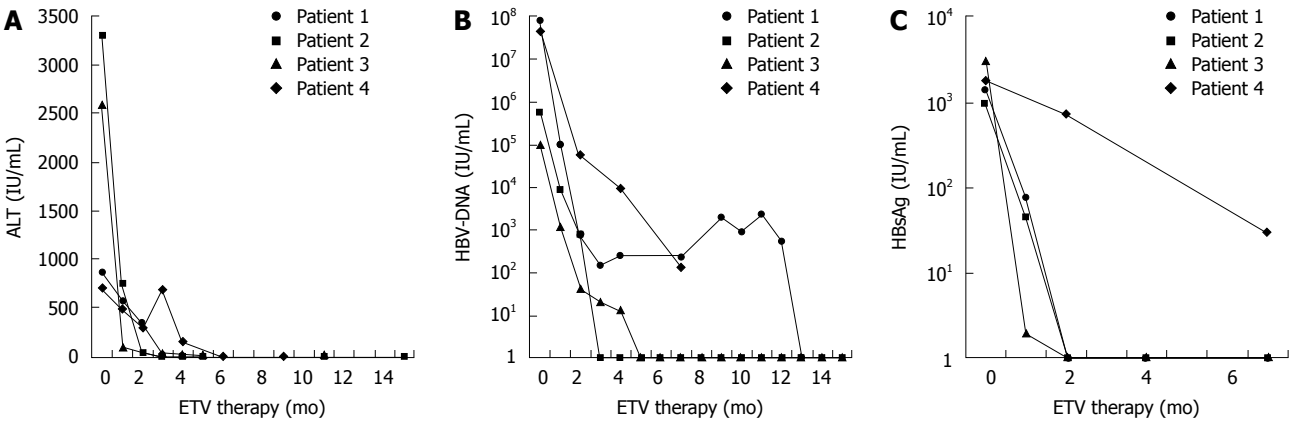

Figure 1 Temporal changes in laboratory values for patients 1-4 at the time of hepatitis B virus reactivation and on entecavir treatment, including: (A) alanine aminotransferase, (B) hepatitis B virus DNA and (C) quantitative hepatitis B surface antigen levels. ALT: Alanine aminotransferase; HBV: Hepatitis B virus; ETV: Entecavir.

tients who developed HBsAg clearance (Figure 1C).

In two of the patients, ETV treatment was withdrawn after 6 mo following HBsAg seroconversion with a follow-up of 6-8 mo after discontinuation. Treatment was well tolerated in all patients and no side effects were observed.

DISCUSSION

HBV-R occurs in patients who have been exposed to HBV and who are receiving immunomodulatory drugs, after solid organ transplantation or HSCT. It has also been described to appear spontaneously[6,7] or in the presence of malignancies. HBV-R can be severe and result in acute hepatic failure and death.

Here, we report four patients with hematological malignancies, who developed HBV-R. In all of these patients, ETV induced suppression of HBV that was associated with rapid clinical improvement, without any side effects. ETV is known to have a very potent antiviral effect that has been shown to be greater than that observed with lamivudine, and has a high resistance barrier[8]. Also, almost 10 years after the introduction of lamivudine, patients are at considerable risk for primary infection with lamivudine-resistant HBV strains, against which, lamivudine treatment is useless. For these reasons, ETV was chosen as initial treatment in our patients.

Drug-induced liver injury[9] or lactic acidosis[10] that have recently been described in patients undergoing treatment with ETV were not observed in our patients. Safe and efficient use of ETV in HBV-R has also been described in one patient from Spain recently[11]. However, we have to point out that none of our patients suffered from severe hepatic failure as defined previously[3] or liver cirrhosis.

It has already been reported that patients with HBV-R

tend to have a higher rate of sustained virological response and higher rates of HBeAg seroconversion than patients who are chronically infected. This was also true for our patients who cleared HBeAg in all cases, and three even experienced HBsAg seroconversion. In Japan, a patient who was receiving chemotherapy for B-cell lymphoma was also treated with ETV for HBV-R and became HBsAg-negative after 2 mo of treatment[12]. The patient from Spain also cleared HBsAg after 6 mo of treatment with ETV[11]. Another patient with B-cell lymphoma in France received ETV as first-line therapy after HBV-R. This patient also had undetectable HBV DNA after 4 mo of treatment and no side effects[11]. It is not stated whether this patient also cleared HBsAg.

In two of our patients, ETV has successfully been withdrawn after anti-HBs had reached levels > 100 IU/mL, and these patients are currently being followed up closely. The third patient has an anti-HBs level < 100 IU/mL and ETV treatment is being continued at present (Table 1).

We also evaluated quantitative HBsAg to study its behavior and possible role in the various phases of HBV-R. In acute hepatitis, the levels of HBsAg are usually > 10 000 IU/mL[13]. The highest level that we found during reactivation was 3031.5 IU/mL. All patients that achieved HBsAg clearance showed at least a 1 $\log_{10}$ drop of HBsAg at wk 4 of treatment with ETV, and at wk 8, quantitative HBsAg was undetectable in all patients that experienced HBsAg seroconversion during follow-up. An early serum HBsAg decline has already been shown to be predictive of sustained virological response during treatment with nucleosid(t)e analogs or pegylated interferon[14-17]. Patient 1 showed a rapid decline in quantitative HBsAg with negativity at wk 8, and normalization of ALT and HBsAg seroconversion during follow-up. Despite this response, HBV DNA reached a plateau around 10^3 IU/mL for 9 mo after an initial decline for 3 mo, and became negative only after 1 year of treatment. This finding implies that quantitative HBsAg might be an additional useful tool to optimize the clinical management of patients with HBV-R.

In one patient, tenofovir was added after 3 mo of ETV treatment. This patient had a high viral load prior to treatment (4.5×10^7 IU/mL). After 3 mo, she had a 4 $\log_{10}$ reduction in viral load and ALT decreased from 711 to 313 IU/mL. As a result of progression of CLL, chemotherapy had to be re-started. When ALT levels under chemotherapy rose to 752 IU/mL, tenofovir was added. However, there was no increase in viral load under chemotherapy, and the presence of known ETV resistance mutations was excluded retrospectively. At this time, she had already cleared HBeAg. ETV treatment was suspended for 8 d because the patient ran out of medication. Thus, incomplete viral suppression was possibly not due to lack of efficacy of ETV but resulted from nonadherence. However, we cannot completely rule out that viral suppression by ETV in this patient was insufficient, as even fulminant hepatic failure despite ETV treatment following HBV-R has been described in a single case[18].

It is important to point out that only one of our patients had HBV screening prior to initiation of immunosuppressive treatment. We cannot prove if the other patients really had true reactivation of hepatitis B. However, all of these patients had normal liver values before the start of immunosuppression. *De novo* infection during immunosuppressive therapy is rather unlikely, therefore, we believe that these patients had either resolved or inactive hepatitis B prior to the initiation of immunosuppression. The fact that these data are not available is a weakness of our study, but reflects the real-life situation. Although it is widely recognized that HBV-R can occur with conventional immunosuppression and newer drugs, in particular rituximab, HBV screening often is still not performed in these patients. Taking into consideration the possibly fatal outcome of HBV-R and the fact that it is preventable, HBV screening in all of these patients is mandatory, especially as it has been described that HBV is more prevalent among patients with hematological malignancies of B lineage, and has been hypothesized to even play a pathogenic role in these patients[19,20].

In the patient that was screened prior to treatment and received lamivudine prophylaxis, prophylaxis had retrospectively been stopped too early. At that time, there was no clear consensus about the duration of prophylactic therapy after cessation of chemotherapy. The new EASL guidelines now recommend that prophylaxis should be continued for at least 1 year after withdrawal of immunosuppressive drugs[2]. Furthermore, we and others[5] have experienced HBV-R in HBsAg-negative/anti-HBc-positive patients. Therefore, it might be considered to extend prophylaxis for HBV-R to this subpopulation.

We believe that our results justify further controlled prospective studies to confirm the efficacy and safety of ETV in a larger number of patients with HBV-R.

COMMENTS

Background

Resolved or inactive hepatitis B virus (HBV) infection usually does not harm the patient. However, if for other medical conditions immunosuppressive therapies need to be initiated in these patients, HBV might reactivate with potentially fatal outcome.

Research frontiers

It is recognized that HBV reactivation (HBV-R) might occur under immunosuppressive therapy. Therefore, screening for HBV is recommended before the initiation of these therapies. However, despite these recommendations, screening often is not performed and significant liver disease might develop. The optimal treatment for these patients in order to avoid death or the need for liver transplantation is currently not known, and no systematic studies have addressed this issue.

Innovations and breakthroughs

The authors have successfully treated patients suffering from HBV-R with entecavir (ETV). In all patients, ETV therapy induced suppression of HBV and rapid clinical improvement without side effects.

Applications

As the optimal treatment strategy for HBV-R is currently unknown, the data presented in this article provide a rationale for prospectively studying the use of ETV for the treatment of liver disease due to HBV-R under immunosuppressive therapies.

Terminology

Immunosuppressive therapy most frequently refers to chemotherapy for malignant tumors or agents used for treatment of autoimmune-mediated diseases such as rheumatic diseases.

Peer review

The authors have explained their experience with ETV in immunosuppressed patients with HBV-R. This paper adds important information to the literature, especially the usefulness of ETV in this field, in which data are sparse.

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BRIEF ARTICLE

Endotics system *vs* colonoscopy for the detection of polyps

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Abstract

AIM: To compare the endotics system (ES), a set of new medical equipment for diagnostic colonoscopy, with video-colonoscopy in the detection of polyps.

METHODS: Patients with clinical or familial risk of colonic polyps/carcinomas were eligible for this study. After a standard colonic cleaning, detection of polyps by the ES and by video-colonoscopy was performed in each patient on the same day. In each single patient, the assessment of the presence of polyps was performed by two independent endoscopists, who were randomly assigned to evaluate, in a blind fashion, the presence of polyps either by ES or by standard colonoscopy. The frequency of successful procedures (i.e. reaching to the cecum), the time for endoscopy, and the need for sedation were recorded. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the ES were also calculated.

RESULTS: A total of 71 patients (40 men, mean age 51.9 ± 12.0 years) were enrolled. The cecum was reached in 81.6% of ES examinations and in 94.3% of colonoscopies (P = 0.03). The average time of endoscopy was 45.1 ± 18.5 and 23.7 ± 7.2 min for the ES and traditional colonoscopy, respectively (P < 0.0001). No patient required sedation during ES examination, compared with 19.7% of patients undergoing colonoscopy (P < 0.0001). The sensitivity and specificity of ES for detecting polyps were 93.3% (95% CI: 68-98) and 100% (95% CI: 76.8-100), respectively. PPV was 100% (95% CI: 76.8-100) and NPV was 97.7% (95% CI: 88-99.9).

CONCLUSION: The ES allows the visualization of the entire colonic mucosa in most patients, with good sensitivity/specificity for the detection of lesions and without requiring sedation.

Key words: Colonoscopy; Diagnosis; Endotics system; Polyps; Sedation

Peer reviewer: Damian Casadesus Rodriguez, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

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INTRODUCTION

Video-colonoscopy is considered the gold-standard for the diagnosis of colonic diseases, including carcinomas and polyps[1]. However, this diagnostic technique presents some limitations, such as invasiveness and patient discomfort, which limit the adherence to programs for the early detection of colon carcinoma[2,3]. When undergoing colonoscopy, patients often require sedation, which may result

in the onset of unintended deep sedation[4]. In addition, standard colonoscopy is associated with various procedural risks, ranging from minor complications[5] to cardiopulmonary events[6,7], colon perforation[8,9], infections[10,11] and, in very rare circumstances, death[12].

Newer diagnostic techniques are therefore advocated to overcome these limitations while maintaining a good diagnostic accuracy[1,13]. While the use of such techniques in clinical practice is starting to emerge, direct head-to-head comparisons between different technologies are still lacking[1].

The endotics system (ES) is a new robotic device composed of a workstation and a disposable probe, which gave promising results in a pilot study for the detection of colonic polyps[14]. We report here the results of a head-to-head comparison of ES and standard colonoscopy in the diagnosis of polyps. To our knowledge, this is the first direct comparison of these techniques.

MATERIALS AND METHODS

Study setting and design

This is a prospective, single-centre study conducted at the Department of Gastroenterology of Pisa University Hospital, from March to August 2009. All patients gave informed consent before inclusion in the trial. The study was conducted in accordance to the Helsinki Declaration (2008 version) and its protocol was approved by the Hospital Ethical Committee.

Eligibility criteria

All adult patients (aged 18-75 years) consecutively seen at our Unit were eligible for this study if they met ≥ 1 of the following criteria: (1) age > 40 years with at least a first-grade relative with a previous diagnosis of colorectal carcinoma or adenoma before he/she was 60 years old; (2) were receiving follow-up evaluations after previous endoscopic polypectomy; and (3) were positive at faecal occult blood test (FOBT), as assessed during screening campaigns. Patients were excluded if they were pregnant, affected by chronic renal insufficiency, active ulcerative colitis or Crohn's disease, bleeding lesions of oesophagus, stomach or small intestine, or had undergone abdominal surgical interventions in the 6 mo period prior to study entry.

The ES

The ES (Era Endoscopy S.r.l., Pisa, Italy) is a new CE-marked (the CE marking certifies that a product has met EU consumer safety, health or environmental requirements) medical device for diagnostic colonoscopy, composed of a workstation and a hand-held console which drives a steerable probe through the colon lumen. A complete description of this device has been provided elsewhere[14]. In this study we used a slightly different ES version from the one used in the previous pilot study (25 cm length in the contracted form and 43 cm in the elongated form, with respect to 23 and 37 cm, respectively, of the previous version).

ES probe is sterile, disposable and soft, in order to allow for adjustment of its shape to colon morphology, and avoid stretching maneuvers to reach the cecum. The probe is composed of a head, a steerable tip, a flexible body (all with 17 mm diameter), a thin tail (7.5 mm diameter and 180 cm length) and a special tank with an electro-pneumatic connector. The head hosts both a vision system, including a camera (110° vision angle) with LED light sources, and channels for water jet and air in order to provide rinsing and suction/insufflation, respectively. The workstation allows the endoscopist to drive the probe using the console and to visualize real-time images on a screen.

The key operations performed by the ES can be summarized as follows: (1) the steering, consisting of an electro-pneumatically driven deflection of the head of the robot (a rotation of 180° can be performed in every direction within a short bending diameter); (2) the elongation of the probe body, visually driven by the endoscopist in order to follow the morphology of the intestine; and (3) the control of rinsing, insufflation and suction. Suction allows the endoscopist to remove liquids from the bowel and convey them to the tank. Insufflation may help unfold the bowel wall in order to have a clearer view of the mucosa.

The motion of advancing the probe through the colon follows a cyclic sequence of steps[14].

Study procedures

All patients underwent a standard preparation to colonoscopy: a fiber-free diet in the seven days preceding the examination and oral administration of phosphate sodium lavage solution (80 mL in 2000 mL of water until evacuation of clear yellowish fluid) on the day before the examination. Both ES examination and standard colonoscopy were performed in each patient on the same day. All procedures were performed by endoscopists with a solid experience (> 500 colonoscopies successfully performed) and after complete training with the ES (> 20 tests on pigs or models). In all patients, the first colonic examination was performed with the ES; after that, the patients underwent standard colonoscopy. This sequence was decided in order to avoid possible alterations of the physiologic features of the colon due to standard colonoscopy. Moreover, the current version of the ES does not allow us to perform polypectomy or biopsies; these procedures, if required, have been performed during the standard colonoscopy. Each patient was examined lying on his/her left side and were later turned to the supine position only if required.

In each single patient, the assessment of the presence of polyps was performed by two independent endoscopists, who were randomly assigned to evaluate, in a blind fashion, the presence of polyps either by ES or by standard colonoscopy. The randomization was performed according to a list of numbers generated by a computer and each operator ignored the results of the evaluation performed by the other examiner.

The colonoscopy was judged successful upon the visual recognition of the ileo-cecal valve by the examiner, confirmed by a third independent endoscopist. The di-

Table 1 Demographic characteristics and indications for colonoscopy

Parameter	Value
Total No. of patients (*n*)	71
Males, *n* (%)	40 (56.3)
Age (yr), mean ± SD, (range)	51.9 ± 12.0 (33-81)
Indication for colonoscopy, *n* (%)	
Follow-up of a previous polypectomy	11 (15.5)
Search for faecal occult blood	21 (29.5)
Familiar history of colon neoplasms	39 (54.9)

Table 2 Operative results observed with the endotics system procedure and with standard colonoscopy (*n* = 71 for each procedure) *n* (%)

Parameter	Endotics system	Standard colonoscopy	*P* value
Procedures reaching cecum	58.0 (81.6)	67.0 (94.3)	0.0300
Complete procedure, mean ± SD (min)	45.1 ± 18.5	23.7 ± 7.2	< 0.0001
Patients requiring sedation	0	14.0 (19.7)	< 0.0001

mensions and sites of the polyps identified during the ES and the standard colonoscopy were recorded. Polyps were then removed and/or biopsies were taken as necessary, according to polyp shape and dimensions. Polyp dimensions were estimated as described by Van Gossum *et al*[15]. Colon cleansing was assessed according to Aronchick's scale and recorded[16]. The time required to perform ES and colonoscopy were also measured. All the patients were contacted 1 and 7 d after the procedures to evaluate the possible onset of adverse events.

Antispasmodic medications were not allowed. Midazolam and meperidine were administered and tailored according to each patient's need. These medications were offered to patients who referred pain during either ES or standard colonoscopy.

Data analysis

All the data were analyzed by descriptive statistics, as appropriate. Comparisons between ES and standard colonoscopy results were performed by Student's paired *t*-test or Fisher's exact test, with a P value < 0.05 considered statistically significant. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the ES were calculated with standard 2 × 2 table analysis.

All statistical analyses were performed with SAS software (SAS Institute, Cary, NC).

RESULTS

A total of 71 patients (40 men; mean age 51.9 ± 12.0 years, Table 1) were included in the study and underwent both ES procedure and standard colonoscopy.

Operative results of the two procedures are summarized in Table 2. Overall, the cecum was reached more frequently with standard colonoscopy (P = 0.03 *vs* ES); moreover standard colonoscopy required a significantly shorter time with respect to ES (P < 0.0001). On the other hand, no patients requested sedation during the ES procedure, while 14 subjects (19.7%) requested the administration of midazolam and meperidine during standard colonoscopy (P < 0.0001).

Table 3 Reasons for incomplete views of the colon during examinations *n* (%)

Reasons	Endotics system	Standard colonoscopy
Anal stenosis	1 (1.4)	0
Sigma stenosis	2 (2.8)	0
Dolicolon	0	1 (1.4)
System failure	3 (4.2)	0
Insufficient length of endoscopic device	1 (1.4)	0
Insufficient cleaning	6 (8.4)	3 (4.2)

Diagnostic accuracy

In total, 14 patients were excluded from the analysis of diagnostic accuracy, as a complete view of the colon was not obtained in them with ES and/or standard colonoscopy (Table 3).

Overall, 14 polyps were detected during ES procedure and 15 were identified during standard colonoscopy. The measured mean diameter of the polyps was comparable with the two procedures (7.64 ± 3.82 mm for ES, 7.50 ± 3.18 mm for standard colonoscopy).

Sensitivity of ES, with respect to standard colonoscopy, was 93.3% [95% confidence interval (95% CI): 68.0-99.0], and specificity was 100% (95% CI: 76.8-100). PPV and NPV were 100% (95% CI: 76.8-100) and 97.7% (95% CI: 88.0-99.9), respectively.

Adverse events

Six patients (8.4%) reported adverse events (nausea, headache, abdominal pain and discomfort). All events were of mild intensity and a spontaneous recovery occurred within 48 h from the onset in all cases. As the onset of adverse events was evaluated 1 and 7 d after the colonoscopy procedures and one single cleaning solution was used, it was not possible to distinguish between adverse events occurring during ES examination and those occurring during standard colonoscopy.

DISCUSSION

Overall, the results of this study suggest that the ES procedure may represent an accurate tool for the detection of colon polyps. Despite the fact that ES was not able to reach the cecum in some cases and was longer than the standard colonoscopy, it had a comparable diagnostic accuracy and did not require the administration of sedating drugs.

It is widely accepted that standard colonoscopy is associated with the possible onset of adverse events and with a low acceptance by patients[2-12]. Newer technologies for colonoscopy are therefore being evaluated in order to over-

come these limitations.Technologies under development for the study of the colon include the Invendoscope™, the Video Capsule Endoscopy and the Aeroscope.

The Invendoscope™ (Invendo Medical, Kissing, Germany) is a single-use colonoscope based on motor driven inverted sleeve technology with a working channel[17]. The results of a single-arm, pilot study on this device conducted on 39 healthy volunteers showed absence of pain in 92% of patients undergoing endoscopy; the cecum was reached in 82% of cases, after a mean time of 23 min[17]. However, no data concerning its diagnostic accuracy are currently available[17].

The Video Capsule Endoscopy (Given Imaging Ltd., Yoqneam, Israel) is a pill-size capsule, activated upon swallowing, which records images of the colonic mucosa[18]. In a pilot study on 41 patients, this device showed a PPV of 59% and a NPV of 84% with respect to standard colonoscopy, with a specificity of 70%[18]. These results were overall confirmed in a larger, recently published multicenter study; however, in this study the sensitivity of Visual Capsule Endoscopy was lower than that associated with standard colonoscopy[15]. Moreover, this device cannot clear colonic debris during the procedure or insufflate air into collapsed intestines[19].

Last, the Aeroscope (GI View Ltd, Ramat Gan, Israel) is a self-propelling, disposable endoscope, that uses low-pressure carbon dioxide to propel a balloon device through the colon, thus facilitating the motion of the colonoscope and reducing discomfort, pain and the risk of perforation[20]. In a study conducted with 12 volunteers, this device reached the caecum in 83% of cases, after a mean time of 14 min. However, 17% of patients requested analgesia, and 33% experienced symptoms consistent with a vagal reaction, including sweating and bloating[20].

The present study lends support to a possible introduction of the ES into clinical practice. In a previous pilot study, conducted in 40 patients, the ES was associated with significantly lower pain intensity and less discomfort, when compared to standard colonoscopy (pain intensity 0.9 *vs* 6.9; discomfort 1.1 *vs* 6.8; both parameters were evaluated on a 1-10 scale)[14]. The high diagnostic accuracy and the lack of need for sedation reported in the present study during the ES procedure may represent further advantages of this technology. In particular, we believe that the reduced need for sedation may have a particular importance: it has been suggested that sedation may be associated with an increase in the onset of cardiopulmonary events and of unintended deep sedation[4,6], although these findings were challenged by a recent meta-analysis[21]. Noteworthy, the ES probe is a single-use device, thus limiting the risk of cross-infections and reducing the overall examination time, since no decontamination is required. Moreover, a single endoscopist may perform the entire procedure, without the need of any assistance by nurses.

However, it must be pointed out that the ES was associated with a lower rate of cecum reach, a more frequent incomplete view of the colon and a longer time to perform the examination than standard colonoscopy. In addition, the current version of this device does not allow the endoscopist to perform polypectomy or biopsies.

We speculate that the higher number of patients with insufficient cleaning during the ES procedure than during the standard colonoscopy may be due to the smaller diameter of the suction device of the ES, when compared to that of the standard video-colonoscopy (1 mm *vs* 3.2 mm, respectively). Moreover, other potential limitations may be also related to low level of training with the ES, since the endoscopist performed > 500 colonoscopies and only > 20 ES in models and pigs with "similar human anatomy".

This study has some limitations that must be acknowledged. First, it was performed in an overall limited number of patients; however, pilot studies with a similar or even smaller sample size have been conducted to evaluate other colonoscopy devices[17,18,20]. Larger studies are required for a more complete evaluation of the ES. Second, inclusion and exclusion criteria were not stringent, potentially limiting the robustness of the analysis; however, this decision was taken in order to better reproduce clinical practice, even in an experimental setting. Third, the ES was compared only with a standard colonoscopic procedure, and not with any other new devices for colonoscopy. However, even if head-to-head comparisons with such devices is still lacking, and the available pieces of evidence do not permit us to retrieve definite findings, the results obtained with the ES may be preliminarily considered at least comparable with those observed with other alternative systems for colonoscopy[17-20].

In conclusion, the ES has shown a high diagnostic accuracy, overall comparable to that reported with standard colonoscopy, and it appeared to be not associated with significant pain/discomfort[14] or with the need for sedation. Although ES seems to show potential shortcomings such as lower cecum intubation rate and/or long duration of endoscopy, it may be considered a promising alterative to standard colonoscopy in the detection of colonic polyps, even if introduction of a tool to perform polypectomy or biopsies is advisable. On this basis, it may be considered a promising alterative to standard colonoscopy in the detection of colonic polyps.

It has been suggested that the introduction of this diagnostic instrument into clinical practice could facilitate the adoption of colonoscopy as first-level screening, with a possible reduction in the incidence of colon cancer-induced mortality[14]. If larger studies, which should also evaluate the optimal bowel preparation conditions and further investigate the need for sedation, will confirm the preliminary evidence collected so far, the ES could play an important role in the detection of colorectal cancer diseases.

COMMENTS

Background

Video-colonoscopy is considered the gold-standard for the diagnosis of colonic diseases, including carcinomas and polyps. However, this diagnostic technique presents some limitations, such as invasiveness and patient discomfort, which limit the adherence to programs for the early detection of colon carcinoma. Furthermore, standard colonoscopy may be associated with various procedural risks.

Research frontiers

Newer diagnostic techniques are advocated to overcome limitations of video-colonoscopy while maintaining a good diagnostic accuracy. The endotics system (ES) is a new robotic device composed of a workstation and a disposable probe, which gave promising results in a pilot study for the detection of colonic polyps. The research hotspot is a head-to-head comparison of ES and standard colonoscopy in the diagnosis of polyps.

Innovations and breakthroughs

The present study lends support to a possible introduction of the ES into clinical practice. The high diagnostic accuracy and the lack of need for sedation reported in the present study during the ES procedure may represent further advantages of this technology.

Applications

The ES has shown a high diagnostic accuracy, overall comparable to that reported with standard colonoscopy, and it appeared to be not associated with significant pain/discomfort or with the need for sedation. It has been suggested that the introduction of this diagnostic instrument into clinical practice could facilitate the adoption of colonoscopy as a first-level screening procedure, with a possible reduction in the incidence of colon cancer-induced mortality.

Terminology

The ES: is a new medical device for diagnostic colonoscopy, composed of a workstation and a hand-held console which drives a steerable probe through the colon lumen. Standard preparation to colonoscopy: a fiber-free diet in the seven days preceding the examination and oral administration of phosphate sodium lavage solution (80 mL in 2000 mL of water until evacuation of clear yellowish fluid) on the day before the examination.

Peer review

The present study described a new device for diagnostic colonoscopy, named "ES". The authors aimed to compare ES with video-colonoscopy in regard to the detection rate of polyps. Moreover, use of sedation, cecum intubation rate and duration of endoscopy were assessed. The authors concluded that ES allowed the visualization of the entire colonic mucosa in most patients, with good sensitivity/specificity for the detection of lesions and without requiring sedation. The study is well-written and designed.

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BRIEF ARTICLE

Incidence of congenital hemolytic anemias in young cholelithiasis patients

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Abstract

AIM: To clarify the incidence of congenital hemolytic anemias (CHA) in young cholelithiasis patients and to determine a possible screening test based on the results.

METHODS: Young cholelithiasis patients (< 35 years) were invited to our outpatient clinic. Participants were asked for comorbidities and family history. The number of gallstones were recorded. Blood samples were obtained to perform a complete blood count, standard Wright-Giemsa staining, reticulocyte count, hemoglobin (Hb) electrophoresis, serum lactate dehydrogenase and bilirubin levels, and lipid profile.

RESULTS: Of 3226 cholecystectomy patients, 199 were under 35 years, and 190 with no diagnosis of CHA were invited to take part in the study. Fifty three patients consented to the study. The median age was 29 years (range, 17-35 years), 5 were male and 48 were female. Twelve patients (22.6%) were diagnosed as thalassemia trait and/or ıron-deficiency anemia. Hb levels were significantly lower (P = 0.046), and mean corpuscular volume (MCV) and hematocrit levels were slightly lower (P = 0.072 and 0.082, respectively) than normal. There was also a significantly lower number of gallstones with the diagnosis (P = 0.007).

CONCLUSION: In endemic regions, for young cholelithiasis patients (age under 35) with 2-5 gallstones, the clinician/surgeon should pay attention to MCV and Hb levels as indicative of CHA.

Key words: Cholelithiasis; Congenital hemolytic anemia; Screening test

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INTRODUCTION

Congenital hemolytic anemias (CHA), especially thalassemia and sickle cell anemia (SCA), are endemic in southern Turkey: the prevalence of the β-thalassemia trait is reported to be 1.84%-2.3% and the prevalence of sickle hemoglobin (Hb) is 4.6%[1,2]. Symptomatic exacerbation of severe anemia can be easily diagnosed. Preventive measures and treatment of complications are well known by the hematologists. However, the thalassemia trait and SCA trait patients rarely present with clinical symptoms, and minor hematological findings may be missed in routine clinical practice.

One of the frequent comorbidities of CHA is gallbladder stones. Chronic hemolysis causes increased bilirubin excretion and gallstone formation[3-5]. The most common types of gallstones associated with CHA are pigment gallstones. Even in cholesterol stones, a bilirubin nidus has been documented[6]. Thus, increased bilirubin acts a trigger in the formation of gallstones regardless of stone type[7]. Eighty percent of SCA and β-thalassemia major patients and more than 40% of spherocytosis patients have cholelithiasis detected at a young age, i.e. younger than 30 years[8]. Cholelithiasis would probably be the first clinical finding for these patients, particularly in hereditary spherocytosis or other rare congenital erythrocyte membrane defect patients.

We recognize that there is a distinct number of young patients with cholelithiasis in our region. Therefore, we aimed to clarify the incidence of known and unknown CHA in young cholelithiasis patients in our region. Our secondary aim was to determine a possible screening test based on the results.

MATERIALS AND METHODS

The study was approved by our university's research committee and ethics committee (KA 07/90). Between 1998 and 2007, 3226 cholelithiasis patients were treated in our hospital. Of these, 199 (6.1%) were younger than 35 years. Their files were analyzed retrospectively. Patients with known CHA were excluded. The rest of the patients were contacted by telephone and asked if they had any known hematological disease. Thereafter they were informed of the study and were invited to the outpatient clinic. Written informed consent of all participants was obtained at the clinic visit. Participants were asked to fill in a short inquiry form, which included height, weight, comorbidities, family history of any hematological disease, medications, and number of childbirths and oral contraceptive use for females. The number of gallstones had also been recorded from the preoperative medical records. Blood samples were then obtained for analysis: complete blood count, standard Wright-Giemsa staining, reticulocyte count, high performance liquid chromatography (HPLC), Hb electrophoresis, serum lactate dehydrogenase and bilirubin levels, and lipid profile.

All parameters of all patients were evaluated by the same hematologist (EK) to determine if there was any diagnosis or suspicion of CHA.

Statistical analyses were performed with SPSS 11 for Windows. In order to analyze categorical variables the chi-square test was used. The Student *t* test and its nonparametric counterpart, the Mann Whitney *U* test, were used for continuous variables. The receiver operating characteristics (ROC) curve for the studied variables and diseased patients was also obtained for sensitivity analysis.

RESULTS

Nine of 199 cholelithiasis patients (4.52%) had a previous diagnosis of SCA. The rest of the 190 patients were invited to the hospital and 53 patients (27.9%) consented to the study. The median age of these 53 patients was 29 years (range, 17-35 years), with 5 males and 48 females.

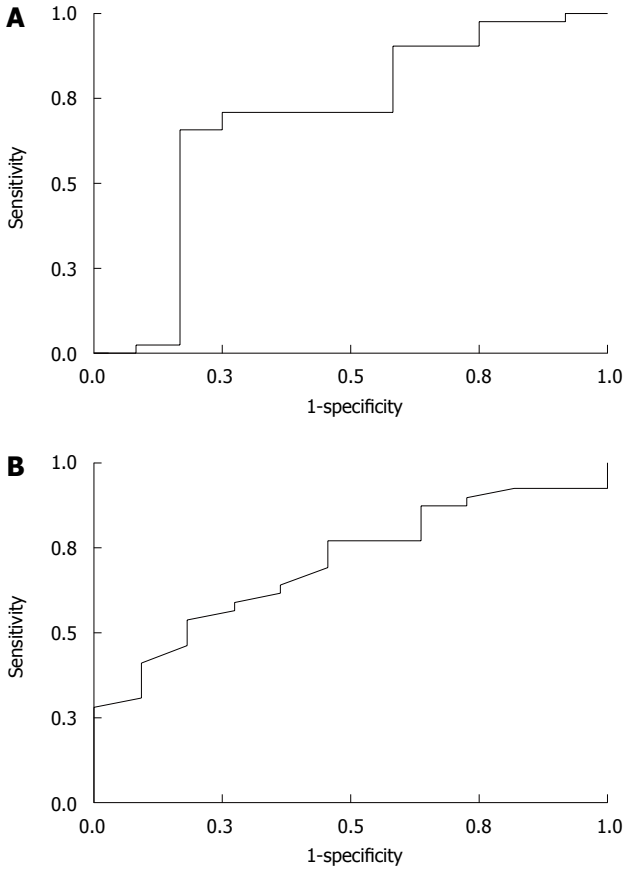

Figure 1 Receiver operating characteristics curve for mean corpuscular volume and α-thalassemia (A), receiver operating characteristics curve for hemoglobin and α-thalassemia (B).

Hematological evaluation of all parameters revealed that there was no patient with SCA or β-thalassemia. However, 12 patients (22.6%) were diagnosed with suspected thalassemia trait and/or ıron-deficiency anemia (IDA).

The mean values of the studied parameters for suspected patients and the patients with normal hematologic findings are shown in Table 1. Hb levels were significantly lower in suspected patients compared to normal (P = 0.046). There was also a trend for lower mean corpuscular volume (MCV) and hematocrit (Hct) in suspected patients (P = 0.072 and 0.082, respectively). There was no significant difference for the other parameters between the groups (Table 1).

ROC curve analysis of MCV, Hb, and Hct for the diagnosis of α-thalassemia were performed separately. MCV and Hb were significantly correlated with the diagnosis of α-thalassemia. The ROC curves of MCV and Hb are shown in Figure 1. For a cutoff MCV of 82 μm^3, and Hb of 120 mg/L, sensitivity, specificity, positive and negative predictive values for diagnosis are shown in Table 2.

Obesity, gender, number of gallstones, MCV and Hb levels (lower or higher than the threshold) were grouped and analyzed to diagnose α-thalassemia. The patient distribution is shown in Table 3. There was a significant difference for the number of gallstones and MCV < 82 μm^3

Table 1 Mean values of blood parameters for suspected patients and patients with normal hematologic findings

Variables	Normal		Suspected		*P*
	No. patients	mean ± SD	No. patients	mean ± SD	
Age (yr)	40	28.8 ± 3.9	12	27.2 ± 5.0	0.356
Total cholesterol (mg/dL)	39	181.5 ± 36.6	11	184.2 ± 34.1	0.886
HDL (mg/dL)	39	46.8 ± 13.9	11	47.0 ± 12.5	0.905
LDL (mg/dL)	39	112.6 ± 33.2	11	117.9 ± 20.82	0.546
Triglyceride (mg/dL)	38	131.2 ± 87.3	10	135.8 ± 57.2	0.916
Total bilirubin (mg/dL)	40	0.62 ± 0.35	10	0.50 ± 0.22	0.214
Indirect bilirubin (mg/dL)	40	0.42 ± 0.26	10	0.37 ± 0.18	0.505
LDH (U/L)	39	135.5 ± 35.0	11	133.5 ± 21.7	0.836
Hemoglobin (g/dL)	38	13.25 ± 1.46	11	12.31 ± 1.16	0.046
Hematocrit (%)	38	39.6 ± 4.2	11	37.2 ± 3.4	0.082
MCV (μm^3)	36	84.32 ± 4.37	11	80.25 ± 5.52	0.072
Reticulocyte (%)	36	1.90 ± 3.02	10	1.37 ± 0.39	0.312
Height (cm)	35	163.2 ± 6.6	8	161.7 ± 6.4	0.589
Weight (kg)	37	69.7 ± 13.4	10	72.2 ± 14.9	0.640
Hb A1 (%)	38	96.16 ± 0.57	11	96.10 ± 0.51	0.757
Hb A2 (%)	38	2.88 ± 0.35	11	2.76 ± 0.41	0.401
Hb S (%)	0	0 ± 0	0	0 ± 0	NA
Hb F (%)	3	1.03 ± 0.32	0	0 ± 0	0.360
BMI (kg/m^2)	33	25.6 ± 4.2	7	27.3 ± 5.6	0.472

HDL: High density lipoprotein; LDL: Low density lipoprotein; LDH: Lactate dehydrogenase; MCV: Mean corpuscular volume; Hb: Hemoglobin; Hb S: Sickle Hb; Hb F: Fetal Hb; BMI: Body mass index: NA: Not applicable.

Table 2 Predictive values of mean corpuscular volume and hemoglobin in determining hemoglobinopathies

Variable	Sensitivity	Specificity	Positive predictive value	Negative predictive value
MCV < 82 μm^3	70.7	75.0	42.8	90.6
Hb < 12 g/dL	82.1	63.6	30.8	80.0

MCV: Mean corpuscular volume; Hb: Hemoglobin.

Table 3 Distribution of patients according to categorical variables *n* (%)

	Diagnosis		Total	*P*
	Normal	Suspected		
BMI (kg/m^2)				
< 25	12 (80.0)	3 (20.0)	15	0.267
25-30	17 (89.5)	2 (10.5)	19	
> 30	8 (61.5)	5 (38.5)	13	
Hemoglobin (g/dL)				
< 12	9 (69.2)	4 (30.8)	13	0.425
⩾ 12	32 (80.0)	8 (20.0)	40	
Gender				
Female	37 (77.1)	11 (22.9)	48	0.883
Male	4 (80.0)	1 (20.0)	5	
MCV (μm^3)				
< 82	12 (57.1)	9 (42.9)	21	0.007
⩾ 82	29 (90.6)	3 (9.4)	32	
No. of gallstones				
Only one	2 (33.3)	4 (66.7)	6	0.004
2-5	4 (66.7)	2 (33.3)	6	
Multiple	35 (85.4)	6 (14.6)	41	
Total	41 (77.4)	12 (22.6)	53	

MCV: Mean corpuscular volume; BMI: Body mass index.

with the diagnosis of thalassemia trait ($P = 0.004$ and 0.007, respectively).

DISCUSSION

The prevalence of gallbladder stone disease is 10%-20% and is increased in the elderly and female population[9,10].

Diagnosis of symptomatic SCA or β-thalassemia is not difficult. In endemic regions, it is especially important to identify carriers of these diseases. There is no cure, and the affected individuals may pass this disease to the next generation. Until gene therapy becomes a reality, the only approaches to the control of CHA are prevention and avoidance. The prevention strategies for CHA include education, carrier screening, genetic counseling, prenatal diagnosis (by amniotic fluid sampling at pregnancy) and selective termination of affected fetuses[11].

In order to reduce the birth rate of CHA in the areas of risk, efforts should be made to persuade couples to consider screening tests before the first pregnancy. For at-risk couples, details of the disorder should be explained to them so that they can make a decision in the context of their unique medical, moral and social situations[12,13]. In a previous study, premarital couples, who may be carriers for hemoglobinopathies were screened in the southern part of the Turkey by hemoglobin electrophoresis. The frequency of sickle Hb was 4.6% and β-thalassemia 2.3%. In 35 of 2113 prospective families, both partners were found to be carriers[2]. However, carrier screening by Hb electrophoresis is an expensive method for mass screening.

We found that 4.5% of young cholelithiasis patients had a known diagnosis of SCA. In this study, there was no patient with abnormal HPLC electrophoresis results sug-

gesting congenital hemoglobinopathies. However, 22.6% of participants (young cholelithiasis patients) were found to have microcytic anemia. Although they did not present with β-thalassemia or SCA, this result suggested that these patients may have thalassemia trait and/or IDA or any other hemoglobinopathy subtypes. It is reported that in some mutations of β-thalassemia and in heterozygous α-thalassemia, Hb A2 is not elevated[14,15]. Serum iron and ferritin levels must be analyzed to differentiate between IDA and thalassemia. Patients with low serum iron levels should be initially treated with oral iron supplement and then should be re-evaluated according to the response to the treatment. Patients with normal iron levels or those with no response to replacement treatment should be assumed as hemoglobinopathy carriers. However, this is a time-consuming expensive method for differentiation.

For hematological evaluation, it is important to differentiate thalassemia and IDA. Demir *et al*[9] suggested that pre-keratocytes and pencil cells, as morphologic features in the Wright-Giemsa staining, are useful in differentiating IDA and thalassemia trait. However, Wright-Giemsa staining is not suitable for daily practice and needs experienced personal for evaluation. There are other indices reported for differentiation between thalassemia trait and IDA, with sensitivity of approximately 100%[16-18]. The most popular index is the ratio of MCV and red blood cell count, the so-called Mentzer Index, which can be automatically calculated with any of the hematology analyzers. This method can be implemented by hospitals and laboratories to flag positive matches and enables screening of population at risk with little to no additional cost[19,20].

Novel data in this study indicates that general surgeons who practice in CHA-endemic regions, should take care in examining young cholelithiasis patients for CHA. Evaluation of complete blood count should be mandatory for these patients. Physicians should be aware of the MCV and Hb level of the young cholelithiasis patient. A MCV $< 82\ \mu m^3$ and Hb < 12 g/dL should alert the physician to the possibility of thalassemia. A family history of anemia should be sought in these patients and they should consult with a hematologist. Further analysis may be necessary.

Another suggestion which may derived from this study is that young patients with less than 5 (not multiple stones or sludge) gallstones should be suspected for the hemoglobinopathies.

In conclusion, in endemic regions, for the young cholelithiasis patients (age under 35) with few gallstones, the clinician/surgeon should pay attention to MCV, Hb level and Mentzer Index.

COMMENTS

Background

Congenital hemolytic anemias (CHA), especially thalassemia and sickle cell anemia (SCA), are a serious problem in endemic regions. One of the frequent comorbidities of CHA is gallbladder stones. Cholelithiasis would probably be the first clinical finding for these patients.

Research frontiers

In order to reduce the birth rate of CHA in the risk areas, prevention strategies for at-risk couples include education, carrier screening, genetic counseling, prenatal diagnosis (by amniotic fluid sampling at pregnancy) and selective termination of affected fetuses.

Innovations and breakthroughs

Premarital couples, who may be carriers for hemoglobinopathies have been screened in the southern part of the Turkey by hemoglobin electrophoresis. However, carrier screening by hemoglobin electrophoresis is an expensive method for mass screening. The most popular index is the ratio of mean corpuscular volume and red blood cell count, the Mentzer Index, which can be automatically calculated with any of the hematology analyzers. This method can be implemented by hospitals and laboratories to flag positive matches and enables screening of the population at risk with little to no additional cost.

Applications

General surgeons, who practice in CHA-endemic regions, should be careful with young cholelithiasis patients in diagnosing CHA. Evaluation of complete blood tests should be mandatory for young cholelithiasis patients.

Terminology

Thalassemia and sickle cell anemia are congenital hemolytic anemias (hemoglobinopathies) which are endemic in some regions of the world.

Peer review

Because the studied area is an endemic region, findings of this study are not generalizable. However the results should be important for the readers from endemic areas of the world. Low participation rate is another limitation of the study.

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BRIEF ARTICLE

Traction-assisted endoscopic mucosal resection for polypectomy in the large intestine

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Abstract

AIM: To evaluate if traction-assisted endoscopic mucosal resection (TA-EMR) is feasible and if it enables *en bloc* resection of colorectal lesions.

METHODS: Seven patients with a total of 12 colorectal adenomas were prospectively enrolled. All lesions were removed by TA-EMR: one hemostatic clip tied to a white silk suture was applied to the base of the lesion to allow traction through the working channel of the colonoscope. A conventional polypectomy snare was mounted over the suture and the lesion was pulled into the snare and resected in one piece.

RESULTS: All 12 lesions (nine sessile) were resected *en bloc* with free lateral and vertical margins by using this novel technique, including five lesions (5/12, 41.6%) in less-accessible positions, where TA-EMR enabled complete visualization of the base before resection. Mean longest lesion and specimen sizes were 9 mm (range: 6-25 mm) and 11 mm in diameter (range: 7-17 mm), respectively. No serious procedure-related complications were observed.

CONCLUSION: TA-EMR through the endoscope using a hemostatic clip and suture material is technically feasible. Visualization of colorectal lesions in less-accessible locations can be improved.

Key words: Traction-assisted; Endoscopic mucosal resection; Colon polyp; Colonoscopy

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Dauser B, Winkler T, Salehi B, Riss S, Beer F, Herbst F. Traction-assisted endoscopic mucosal resection for polypectomy in the large intestine. *World J Gastroenterol* 2010; 16(43): 5462-5466 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i43/5462.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i43.5462

INTRODUCTION

Endoscopic mucosal resection (EMR) for removal of neoplastic gastrointestinal lesions and early cancers has been established as a minimally invasive technique. Referring to the "adenoma-adenocarcinoma sequence", benign adenomas are regarded as precursors of cancer and should be completely removed during endoscopic procedures[1].

Besides the conventional inject-lift-cut EMR, i.e. strip biopsy, several variations have been developed such as cap-assisted EMR or EMR using a ligating device to enable safe and complete resection with the polypectomy snare[2-5]. *En bloc* resection with different EMR techniques is limited to adenomas of 15-20 mm in diameter[6-8].

However, smaller lesions, and particularly those in less-accessible locations, are sometimes hard to be ensnared and are challenging to the endoscopist. To improve visualization and make *en bloc* resection easier by exerting traction on the lesions while performing EMR or endoscopic submucosal dissection (ESD), several techniques have been described, such as sinker-assisted ESD, percutaneous traction-assisted EMR, magnetic anchor devices, external grasping forceps, thin endoscope-assisted ESD and the S-O-clip device[9-15]. Jeon *et al*[16] recently have reported peroral traction-assisted ESD for treatment of gastric neoplasms. In this paper, we describe a novel technique for traction-assisted (TA)-EMR through the endoscope, and its early results in treating colorectal lesions.

MATERIALS AND METHODS

Between July 2009 and March 2010, seven patients with a total of 12 colorectal lesions were enrolled. There were four female and three male patients, with a mean age of 75 years (range: 61-79 years). All patients had previous endoscopic investigation with histologically confirmed neoplastic adenomas. Each patient had at least one adenoma, which could not be removed by referring gastroenterologists because of its size or location, therefore, patients were sent to our department. We consecutively used the TA-EMR technique for all lesions found during colonoscopy that were > 5 mm in size. Study approval was given by the local ethics committee and written informed consent was obtained from all patients. The study was registered at ClinicalTrials.gov under NCT00966420.

Bowel preparation was done using 3 L polyethylene glycol solution. Colonoscopy was performed with a narrow band imaging video endoscope (CF-H180AI; Olympus, Hamburg Germany). All patients were initially sedated with midazolam (3-5 mg); propofol boluses of 20 mg were given intermittently to maintain sedation.

Using a white silk suture (Freka® PEG Gastric Set; Fresenius, Bad Homburg, Germany), a loop was tied and mounted between the branches of the hemoclip (Figure 1). Normal saline solution (2-10 mL, mean: 2 mL) was injected into the submucosal layer to lift the lesion from the muscular layer in the case of sessile adenomas. A reusable hemoclip-applicator device (HX-5LR-1, Olympus) or a Resolution® Clip (Boston Scientific, Ratingen, Germany) was inserted with the tied suture into the working channel, and the clip was anchored within healthy mucosal tissue at the base of the lesion (Figure 2A and B). After removal of the hemoclip-applicator device, an electrosurgical snare (AS-1-S, ASH-1-S; Cook Medical, Moenchengladbach, Germany) was mounted over the suture and brought into the working channel (Figure 3). At the tip of the video endoscope, the snare was delivered and placed over the lesion, which was pulled into the snare towards the endoscope while closing the snare (Figure 4A). Resection was performed using forced coagulating current, output power 60 W (ICC 200; ERBE, Tuebingen, Germany). Resected specimen secured to the suture material was easily removed, together with the endoscope (Figure 4B). The remaining mucosal defect was carefully inspected using narrow band imaging (NBI). Closure of the resection site was not performed routinely.

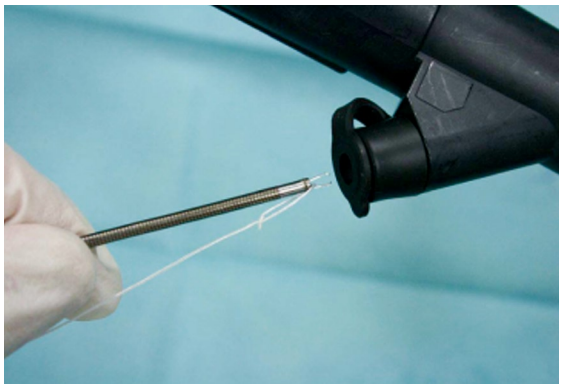

Figure 1 White silk suture loop mounted between the branches of the hemoclip.

All specimens were embedded in paraffin wax. Hematoxylin-eosin-stained sections were evaluated by one pathologist (Beer F) who specializes in gastrointestinal pathology.

RESULTS

Details of the polypectomies are shown in Table 1. Altogether, we removed 12 lesions located in the upper rectum (n = 1), at the rectosigmoid junction (n = 1), in the left (n = 8) or right (n = 1) hemicolon, and at the hepatic flexure (n = 1). Seven small adenomas of 6-9 mm diameter (five sessile) and five large adenomas measuring 10-25 mm (four sessile) were included. On five occasions (5/12, 41.6%), the whole extent and the base of the lesion could not be seen due to the adverse position behind a mucosal fold or at an intestinal bend until traction was exerted. All lesions were resected *en bloc* without any evidence of residual neoplastic tissue using NBI. In addition, histological evaluation confirmed free lateral and circumferential margins (12/12). Advanced pathology, i.e. tubulo-villous adenoma or high-grade dysplasia was found in nine adenomas (9/12, 75%). No neoplastic but hyperplastic tissue was detected once. Mean longest lesion and specimen diameters were 9 mm (range: 6-25 mm) and 11 mm (range: 7-17 mm), respectively. The mean procedure time, starting with injection in the case of sessile adenomas until completion of resection, was 6 min (range: 5-12 min).

No serious procedure-related complications were observed. In one patient (1/12, 8.3%), immediate bleeding after resection had to be controlled using hemostatic clips. All patients were discharged within 1 d after intervention. No delayed complications were observed within 4 wk, when patients were contacted for a final clinical examination.

DISCUSSION

For all neoplastic colorectal lesions, *en bloc* removal is desirable as it enables accurate evaluation of completeness

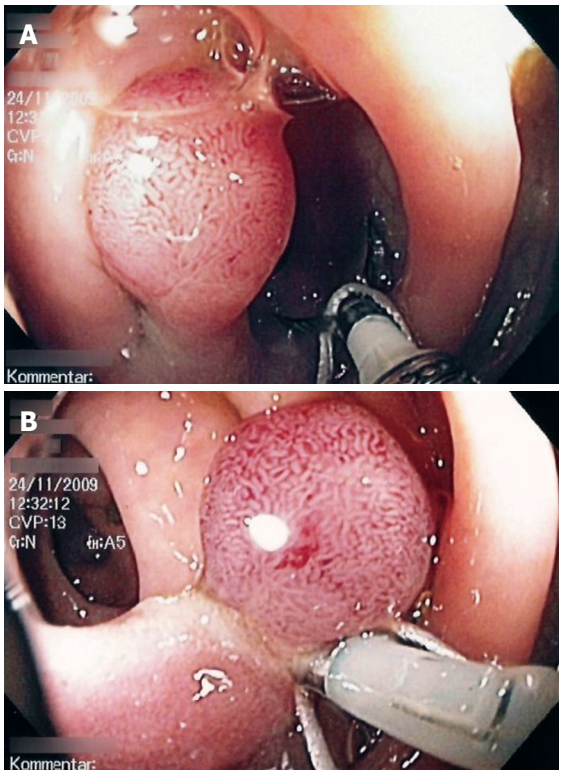

Figure 2 Introduction (A) and application (B) of the clip to the base of the polyp.

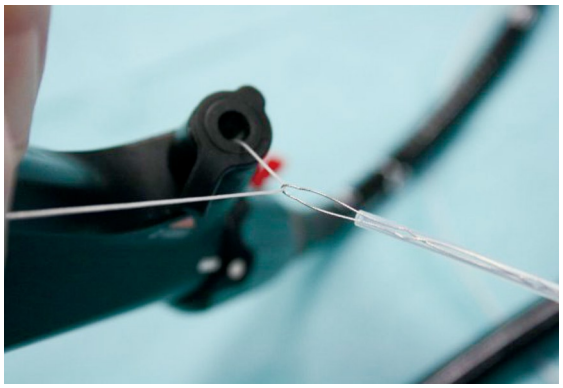

Figure 3 Snare mounted over the suture.

of the resected specimen. Moreover, small adenomas might also carry an increased risk of dysplasia, advanced pathology or even carcinoma[17-20].

En bloc or "single piece" snare polypectomy is regarded as the procedure of choice for polyps larger than 5 mm and up to 15-20 mm. For larger lesions, piecemeal resection or ESD has to be carried out. Despite a smaller size, visualization of lesions in less-accessible locations, e.g. behind mucosal folds or at bends in the bowel, may be hampered. Therefore, exact polypectomy snare placement, particularly in sessile lesions, may be aggravated or even impossible. There are only a few studies that have addressed this problem.

Yoshikane *et al*[21] have described a technique that uses distal attachment to the endoscope to press the semilunar

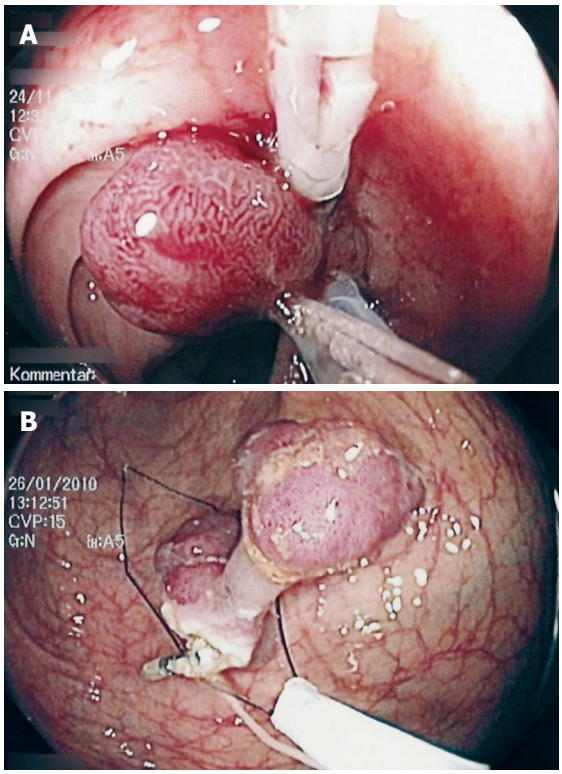

Figure 4 Ensnared polyp (A), polyp retrieval (B).

fold at the anal side of the lesion, to bring the base of the polyp within the visual field. Frimberger *et al*[22] have reported 15 patients treated with polypectomy for problematic polyps, by using a side-viewing duodenoscope as a therapeutic option when polyps were not accessible with the conventional approach. A "suction pseudopolyp technique" for small, flat, non-polypoid lesions of the colon and rectum was published by Pattullo *et al*[23] 2009. This referred to the fact that ensnaring of such lesions may be technically difficult because of minimal or no protruding tissue.

Achievement of good, direct visualization of the base of the lesion is of utmost importance when performing snare polypectomy, to decrease the risk of incomplete resection and complications, such as perforation. Recently, for ESD, different devices and traction systems have been invented to facilitate the resection procedure as mentioned above[10-15]. These various traction methods have their own unique limitations[16].

Our study aimed to evaluate the feasibility of TA-EMR through the endoscope to allow *en bloc* resection of colorectal lesions. Thereafter, we studied if visualization of colorectal adenomas in less-accessible positions can be improved. The technique described uses commonly available and economical materials and can be performed by a single endoscopist using a flexible endoscope with one working channel. Preparation of the hemoclip by mounting a tied loop of a white silk suture between the branches of the clip is easy. Introduction of the clip device and application of the clip to the base of the lesion is not complicated by the suture material.

Table 1 Details of polypectomies

Polyp characteristics	Polyp location	Polyp size	Specimen size (base)	Adverse position	Injection NaCl (mL)	Time (min)	Histology	Grade of dysplasia
Protruded	Descending colon	9	11	No	0	7	Tubulovillous adenoma, R0	Intermediate
Sessile	Descending colon	11	17	No	10	6	Tubular adenoma, R0	High
Sessile	Hepatic flexure	10	15	Yes	2	6	Tubulovillous adenoma, R0	Low
Sessile	Sigmoid colon	12	16	Yes	5	12	Tubulovillous adenoma, R0	Intermediate
Sessile	Sigmoid colon	6	7	No	2	10	Tubulovillous adenoma, R0	Low
Protruded	Sigmoid colon	8	9	No	0	6	Tubulovillous adenoma, R0	Low
Sessile	Rectum, upper third	8	10	No	2	5	Tubular adenoma, R0	Low
Sessile	Ascending colon	9	12	No	4	7	Tubulovillous adenoma, R0	Low
Sessile	Sigmoid colon	6	7	No	2	6	Tubular adenoma, R0	Low
Protruded	Sigmoid colon	25	11	Yes	0	6	Tubulovillous adenoma, R0	High
Sessile	Sigmoid colon	10	15	Yes	2	8	Tubulovillous adenoma, R0	Low
Sessile	Recto-sigmoid junction	7	9	Yes	2	6	Hyperplastic adenoma, R0	/

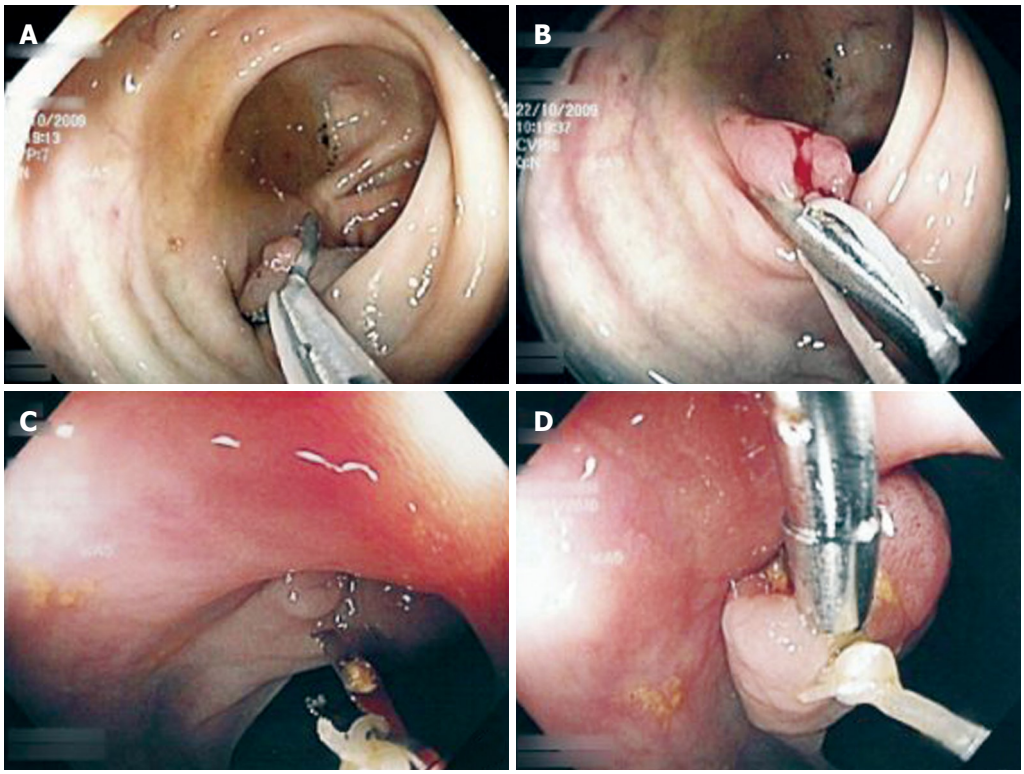

Figure 5 Flat adenoma (A, B) and protruded polyp (C, D) at colonic bend before and after application of traction.

By application of traction, the position of the polyps can be improved for ensnaring and especially the whole amount of lesions and the base can be better visualized (Figure 5). Thereafter, the TA-EMR technique can be used to generate a "pseudo-stalk", and placement of the snare in an adequate distance to the neoplastic tissue is possible.

The materials used are not designed for such an application, therefore, there are some limitations. Assembly of the polypectomy snare over the suture material and bringing it through the working channel can be difficult because the suture has to be gently fixed by the endoscopist at the handle of the endoscope, and the movement of the snare is slightly hindered because of the higher friction due to the suture material in the working channel. Even if it did not happen during this study, there is the possibility that the snare becomes entangled with the clip, or the clip is pulled out of the lesion by excessive traction. We tested clip application *ex vivo* before starting the study. In the case of clip dislodgment, we suggest removal of the endoscope from the bowel; pulling the (potentially crosswise oriented) clip through the working channel may cause damage to the video endoscope. TA-EMR through the endoscope is technically challenging and positioning the snare over the lesion and the clip sometimes can be awkward. Fixation of the suture material to the end of the clip (instead of between the branches) could facilitate this procedure, but it is presently impossible with the available clip devices. A rigid system instead of the suture material would be helpful because it enables a broader range of movements instead of simple traction.

In conclusion, our preliminary limited experience with TA-EMR through the endoscope is promising. Complete *en bloc* resection of colorectal lesions is feasible with the TA-EMR technique, and visualization of adenomas in less-accessible positions can be improved.

COMMENTS

Background

Endoscopic mucosal resection (EMR) for removal of neoplastic colorectal lesions has become established as minimally invasive therapy. Visualization of lesions in less-accessible locations and complete *en bloc* resection, especially of flat adenomas, can be challenging or even impossible.

Research frontiers

Several elaborated techniques have been described to overcome these technical problems, which necessitates special equipment.

Innovations and breakthroughs

Traction-assisted EMR (TA-EMR) uses commonly available and economic materials. Traction is exerted through the working channel of the endoscope, which protects the bowel against the suture material and allows controlled traction by the endoscopist.

Applications

Theoretically, TA-EMR can be performed in every endoscopy unit. Due to the fact that the materials used are not designed for such an application, the technique is challenging. A rigid system instead of the suture material would ease the procedure and allow a broader range of movements.

Terminology

EMR enables removal of neoplastic lesions in one piece by ensnaring adenomas with a polypectomy snare. Lesions at bends in the bowel or behind mucosal folds potentially cannot be ensnared. To bring adenomas to a better position for EMR, traction can be exerted to allow complete visualization and *en bloc* resection.

Peer review

There is an obvious interest in ways to remove difficult polyps, particularly those that lie behind colonic folds. A number of methods have been described to remove these polyps but, thus far, none has been entirely satisfactory. This report describes a relatively simple traction method.

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BRIEF ARTICLE

Increased osteopontin and liver stiffness measurement by transient elastography in biliary atresia

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Abstract

AIM: To analyze plasma osteopontin levels and liver stiffness using transient elastography in postoperative biliary atresia (BA) children compared with healthy controls.

METHODS: Thirty children with postoperative BA and 10 normal controls were enrolled. The patients were categorized into two groups according to their jaundice status. Plasma levels of osteopontin were determined using commercially available enzyme-linked immunosorbent assay. Liver stiffness was measured by using transient elastography (Fibroscan). Ten validated Fibroscan measurements were performed in each patient and control with the result expressed in kilopascals (kPa).

RESULTS: Plasma osteopontin was significantly elevated in BA children compared with that of healthy controls (47.0 ± 56.4 ng/mL *vs* 15.1 ± 15.0 ng/mL, $P = 0.01$). The liver stiffness measurement was markedly elevated in the patients with BA compared with that of controls (26.9 ± 24.6 kPa *vs* 3.9 ± 0.7 kPa, $P = 0.001$). Subgroup analysis showed that the BA patients with jaundice had more pronounced plasma osteopontin levels than those without jaundice (87.1 ± 61.6 ng/mL *vs* 11.9 ± 6.1 ng/mL, $P = 0.001$). Furthermore, the mean liver stiffness was significantly greater in the jaundiced BA patients compared with non-jaundiced patients (47.7 ± 21.8 kPa *vs* 8.7 ± 3.0 kPa, $P = 0.001$). Additionally, plasma osteopontin was positively related to serum total bilirubin ($r = 0.64$, $P < 0.001$). There was also a correlation between plasma osteopontin and liver stiffness values ($r = 0.60$, $P < 0.001$).

CONCLUSION: High plasma osteopontin positively correlated with degree of hepatic fibrosis and could be used as a biochemical parameter reflecting disease severity in postoperative BA children.

Key words: Biliary atresia; Fibroscan; Jaundice; Liver stiffness; Osteopontin

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INTRODUCTION

Biliary atresia (BA) is an intractable liver disease of unknown cause that affects hepatic bile ducts resulting in chronic cholestasis, hepatic fibrosis and biliary cirrhosis. BA children mostly present with a triad of obstructive jaundice, acholic stools, and hepatosplenomegaly. It is the most common indication for liver transplantation in infants and children[1]. Even after effective bile flow has been established by the Kasai operation, the disease still progresses to end-stage liver disease, in which patients will suffer from the complication of portal hypertension and hepatic dysfunction[2,3]. Several etiologies have been considered to account for the pathogenesis of BA, including neonatal viral infections, genetic insults, and abnormalities in immune response, but the precise mechanism of BA is poorly understood[4]. Although it has been documented that a variety of cytokines and growth factors, including bone morphogenetic protein 7[5], basic fibroblast growth factor[6], and stem cell factor[7], play essential roles in the pathophysiology of BA, published data regarding osteopontin expression in postoperative BA is currently limited.

Osteopontin, also known as early T-cell activation gene-1 (Eta-1), is a secreted phosphoprotein 1 (SPP1) that has been implicated in the pathogenesis of various inflammatory and fibrotic disorders. It stimulates T cell proliferation and induces T cells and macrophages to express other T helper type 1 (Th1) cytokines during inflammation[8]. Osteopontin also induces accumulation of extracellular matrix by binding to type I collagen, fibronectin, and osteocalcin, contributing to tissue fibrosis[9,10]. Osteopontin comprises multiple functional domains, with a high sialic acid content, an aspartate-rich domain, calcium-binding domain, thrombin cleavage site, and many residues with consensus for phosphorylation as well as an integrin-binding arginine-glycine-aspartate (RGD) motif, which play a key role in several inflammatory disorders[11]. High levels of circulating osteopontin have been demonstrated in patients with multiple sclerosis[12], osteoarthritis[13], hepatitis C viral infection[14], as well as gastric and liver cancer[15-19]. However, no detailed studies are presently available regarding the relationship between plasma osteopontin levels and the degree of liver stiffness in BA.

It has been recently reported that measurement of liver stiffness using transient elastography or Fibroscan (Echosens, Paris, France) reflects the degree of hepatic fibrosis, which is a principal factor determining the functional liver reserve[20]. In this study, we postulated that elevated plasma osteopontin levels might be associated with the severity of clinical outcomes and the liver stiffness in BA patients. Hence, the objective of this investigation was to analyze plasma osteopontin levels in postoperative BA patients and to evaluate the possible correlations of plasma osteopontin with the disease severity.

MATERIALS AND METHODS

Patients

This investigation was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, and was conducted in agreement with the Declaration of Helsinki. All parents of children with BA and healthy controls were informed of the study's objectives, and written informed consent was obtained from the parents prior to the children entering the study.

A total of 30 BA patients after Kasai procedure (13 males and 17 females; mean age 7.2 ± 3.4 years) who attended the follow-up visit at the Pediatric Liver Clinic, and 10 healthy children (4 males and 6 females; mean age 7.4 ± 3.9 years) from the Well Baby Clinic at King Chulalongkorn Memorial Hospital, were enrolled in this study. Children in the healthy control group had normal physical examinations.

Among the 30 BA patients in the present study, none had any symptoms and signs of infection or ascending cholangitis or clotting abnormalities at the time of blood sampling. None had received liver transplantation. To compare the clinical outcomes among BA patients, they were categorized according to their levels of serum total bilirubin (TB) into two groups: patients without jaundice (TB < 20 mg/L, n = 16) and patients with persistent jaundice (TB ⩾ 20 mg/L, n = 14).

Liver stiffness measurement

Liver stiffness was measured using Fibroscan (Echosens, Paris, France), a new medical device based on elastometry (or one-dimensional transient elastography). This technique is used to quantify hepatic fibrosis in a totally non-invasive and painless manner, with no contraindications. Details of the technique and the examination procedure have been described in a previous report[21]. Briefly, this system is operated with a probe, with an ultrasonic transducer mounted on the axis of a vibrator. A vibration of mild amplitude and low frequency is transmitted from the vibrator on to the tissue by the transducer itself, which induces propagation of an elastic shear wave through the tissue. A pulse-echo acquisition is performed at this time to follow the propagation of the shear wave and measure its velocity. This velocity is directly correlated to the stiffness of liver, which reflects the degree of fibrosis. Results are expressed in kilopascals (kPa).

All measurements were performed according to the manufacturer's instructions. Patients were placed in supine decubitus position with the right arm in abduction. The probe was placed on the skin between 2 ribs at the level of the right lobe of the liver. The measurement area was located by A-mode images provided by the probe trans-

ducer. Ten validated measurements were performed for each patient. The median value was considered as representative of liver stiffness. A set of measurements was considered to be reliable if the success rate was at least 60% and the interquartile range was less than one third of the median liver stiffness value. Because normal values have not yet been established in healthy children without liver disease, a control group of 10 healthy gender- and age-matched children was recruited. Measurements were performed by the same operator under the same conditions as for the BA patients.

Laboratory tests

Samples of peripheral venous blood were collected from each patient and healthy control, centrifuged, and then stored at -80℃ until analysis. Plasma osteopontin concentrations were measured using a commercially available enzyme-linked immunosorbent assay (Immuno-Biological Laboratories Co., Gunma, Japan) following the manufacturer's recommendations. In brief, standards of recombinant human osteopontin and plasma samples were added to microtiter plates pre-coated with rabbit polyclonal antibody against osteopontin and incubated for 1 h at room temperature. The wells were then washed 7 times with washing buffer and incubated for 30 min at 4℃ with a horseradish peroxidase-labeled mouse monoclonal antibody to human osteopontin. After washing thoroughly with washing buffer for 9 times, substrate solution was added to each well, and the plate was incubated for 30 min at room temperature in the dark. Finally, the reaction was stopped with the stop solution, and then absorbance was measured at 450 nm using an automated microtiter plate reader. Recombinant human osteopontin was used to generate a linear standard calibration curve (range 5-320 ng/mL). In addition, liver function tests, including TB, direct bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), were assessed using a Hitachi 912 automated chemical analyzer at the central laboratory of our hospital. The aspartate aminotransferase to platelets ratio index (APRI) was calculated as follows: (AST/upper limit of normal) × 100/platelet count (10^9/L)[22].

Statistical analysis

Statistical analysis was performed using the statistical package for social sciences (SPSS) software, version 16.0 for Windows. All values are expressed as a mean ± SD. Comparisons of demographic data and biochemical parameters between groups were determined by unpaired *t*-test. Correlations between numerical data were acquired using the Pearson correlation coefficient (*r*). A *P*-value < 0.05 indicated statistical significance.

RESULTS

Comparisons between BA patients and healthy controls

Plasma osteopontin levels were measured in 30 BA patients and 10 healthy controls. There were no statistically significant differences with respect to age (7.2 ± 3.4 years *vs* 7.4 ± 3.9 years) and gender (male:female, 13:17 *vs* 4:6) between the BA patients and healthy controls. The baseline characteristics of the BA patients and healthy controls are summarized in Table 1. Mean plasma osteopontin concentration of BA children was significantly higher in comparison with that of healthy controls (47.0 ± 56.4 ng/mL *vs* 15.1 ± 15.0 ng/mL, $P = 0.01$) (Figure 1A). Furthermore, the mean liver stiffness value was markedly elevated in the patients with BA compared with that of healthy controls (26.9 ± 24.6 kPa *vs* 3.9 ± 0.7 kPa, $P = 0.001$).

Table 1 Biliary atresia patient characteristics (mean ± SD)

Variables	Controls (n = 10)	BA patients (n = 30)	*P*-value
Age (yr)	7.4 ± 3.9	7.2 ± 3.4	0.8
Gender (M:F)	4:6	13:17	0.5
Albumin (g/L)	-	43 ± 0.5	NA
Total bilirubin (mg/L)	-	28 ± 4.3	NA
Direct bilirubin (mg/L)	-	22 ± 4.3	NA
AST (IU/L)	-	129.4 ± 100.8	NA
ALT (IU/L)	-	119.4 ± 105.1	NA
ALP (IU/L)	-	469.9 ± 345.6	NA
Platelet count (10^3/mm^3)	-	167.2 ± 96.3	NA
APRI	-	2.9 ± 2.9	NA
Osteopontin (ng/mL)	15.1 ± 15.0	47.0 ± 56.4	0.01
Liver stiffness (kPa)	3.9 ± 0.7	26.9 ± 24.6	0.001

BA: Biliary atresia; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; APRI: Aspartate aminotransferase to platelets ratio index; NA: Not applicable.

Table 2 Comparison of biliary atresia patients without and with jaundice (mean ± SD)

Variables	BA patients without jaundice (n = 16)	BA patients with jaundice (n = 14)	*P*-value
Age (yr)	7.3 ± 3.7	7.0 ± 3.1	0.9
Gender (M:F)	7:9	6:8	0.5
Albumin (g/L)	46 ± 0.3	39 ± 0.4	0.001
Total bilirubin (mg/L)	5 ± 0.2	55 ± 5.5	0.001
Direct bilirubin (mg/L)	2 ± 0.1	46 ± 5.5	0.003
AST (IU/L)	64.9 ± 46.9	203.1 ± 95.5	0.001
ALT (IU/L)	73.6 ± 61.2	171.8 ± 121.5	0.01
ALP (IU/L)	287.9 ± 116.8	677.9 ± 404.1	0.001
Platelet count (10^3/mm^3)	215.8 ± 81.3	111.6 ± 81.0	0.002
APRI	0.8 ± 0.8	5.1 ± 2.7	< 0.001
Osteopontin (ng/mL)	11.9 ± 6.1	87.1 ± 61.6	0.001
Liver stiffness (kPa)	8.7 ± 3.0	47.7 ± 21.8	0.001

BA: Biliary atresia; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; APRI: Aspartate aminotransferase to platelets ratio index.

Comparisons between BA patients with and without persistent jaundice

The demographic data and biochemical parameters including liver function tests, plasma osteopontin levels and liver stiffness values based on jaundice status are given in Table 2.

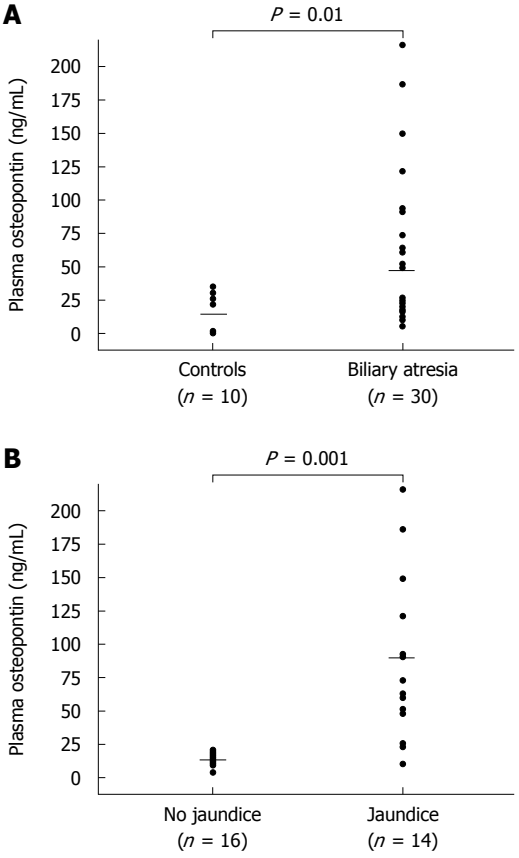

Figure 1 Plasma osteopontin levels. A: In biliary atresia (BA) patients and healthy controls; B: In BA patients without and with jaundice.

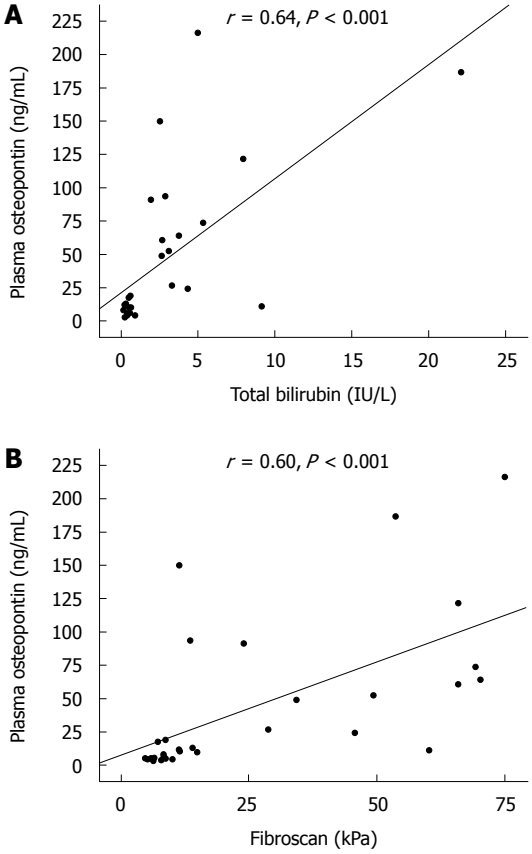

Figure 2 Correlation analysis in biliary atresia patients. A: Correlation between plasma osteopontin and serum total bilirubin in biliary atresia (BA) patients ($r = 0.64$, $P < 0.001$); B: Correlation between plasma osteopontin and liver stiffness using Fibroscan in BA patients ($r = 0.60$, $P < 0.001$).

Subgroup analysis demonstrated that BA patients with persistent jaundice had significantly higher serum levels of AST, ALT, ALP, and APRI than those without jaundice. As shown in Figure 1B, the BA patients with persistent jaundice had more pronounced plasma osteopontin levels than those without jaundice (87.1 ± 61.6 ng/mL *vs* 11.9 ± 6.1 ng/mL, $P = 0.001$). There was no significant difference in plasma osteopontin between BA patients without jaundice and healthy controls. In addition, the mean liver stiffness was significantly greater in the jaundiced BA patients compared with non-jaundiced patients (47.7 ± 21.8 kPa *vs* 8.7 ± 3.0 kPa, $P = 0.001$). Moreover, plasma osteopontin was positively related to serum TB ($r = 0.64$, $P < 0.001$) (Figure 2A). There was also a correlation between plasma osteopontin and liver stiffness score ($r = 0.60$, $P < 0.001$) (Figure 2B).

DISCUSSION

BA is one of the most serious digestive tract disorders characterized by progressive, fibrosclerotic cholangiopathy affecting both intrahepatic and extrahepatic bile ducts. It may lead to obstruction or discontinuity of the biliary tract at any point between the porta hepatis and the duodenum. Without medical and surgical treatment, the majority of BA patients will develop severe cholestasis, hepatic fibrosis, and eventually die within a few years[1].

It is accepted that Kasai hepatoportoenterostomy is the first line of surgical treatment. Despite early diagnosis and successful Kasai operation, more than half of the BA patients inevitably develop biliary cirrhosis, portal hypertension, and end-stage liver disease[2]. Alternatively, liver transplantation is an effective treatment modality when the Kasai hepatoportoenterostomy has failed and serious complications occur such as recurrent ascending cholangitis, persistent jaundice, cirrhosis, progressive ascites, and bleeding esophageal varices[3]. Nonetheless, the exact pathophysiology of liver fibrosis or cirrhosis in BA children remains unknown.

Osteopontin is a highly phosphorylated acidic glycoprotein that is not only present in bone, but also is produced by a number of cell types including chondrocytes, immune cells, smooth muscle cells, epithelial cells, and endothelial cells[23,24]. It is now considered as a potent chemo-attractive and pro-inflammatory mediator which is involved in a variety of physiologic and pathologic events, such as cell adhesion, proliferation, migration, inflammation, apoptosis, vascular remodeling, and wound healing[25,26]. The role of osteopontin in liver diseases has not been completely elucidated. Carbon tetrachloride administration has been shown to increase osteopontin expression in the rat liver where it was localized mostly

to activated Kupffer cells, hepatic macrophages, and stellate cells[27,28]. Recombinant osteopontin also activated rat hepatic macrophage migration *in vitro*, whereas *in vivo*, the levels of macrophage infiltration in injured liver were reduced in osteopontin-knockout mice[28]. These findings indicate that osteopontin may be involved in the initiation of inflammatory reactions in the liver and activation of hepatic fibrosis.

In recent years, the upregulation of osteopontin expression has been documented by using gene expression microarray and Northern blot analysis of liver samples from BA patients[29]. It has been shown in previous studies that gene expression of hepatic osteopontin is highly upregulated in BA. This finding suggests that human bile duct epithelial cells are able to synthesize osteopontin[30,31]. These observations prompted us to speculate that osteopontin may be responsible for the pathogenesis of BA. However, plasma osteopontin level at various clinical stages of BA and its possible role in BA patients has not received much attention.

In the present study, we demonstrated that plasma osteopontin levels in BA patients were significantly elevated compared to those in healthy controls. Further analysis revealed that plasma osteopontin levels were markedly higher in BA patients with persistent jaundice than those without jaundice. High plasma osteopontin was positively correlated with serum TB in postoperative BA patients. These findings suggest that plasma osteopontin is associated with jaundice status in postoperative BA patients. Additionally, jaundice status in BA patients is likely to be a parameter for intrahepatic biliary obstruction. Hence, these results suggest that osteopontin plays a possible role in the pathogenesis of hepatocellular damage in BA, and that it seems to be correlated with the degree of biliary obstruction.

To the best of our knowledge, this study is the first to show that plasma osteopontin is elevated in BA patients compared with healthy controls, and that osteopontin concentration is associated with clinical outcome (status of jaundice, hepatic dysfunction, and hepatic fibrosis) in BA. Increased plasma osteopontin has been documented in a variety of liver disorders, including acute hepatic dysfunction, chronic hepatitis, liver cirrhosis, hepatocellular carcinoma, and primary biliary cirrhosis[16-19]. In accordance with our study, plasma osteopontin was shown to be significantly elevated in cirrhosis patients, and was correlated with the severity of hepatic injury[17]. Kim *et al*[19] also revealed an elevation of plasma osteopontin with advancing degree of hepatocellular carcinoma. These findings suggest that high plasma osteopontin is associated with hepatic damage and hence reflects liver dysfunction. Accordingly, our results also showed that plasma osteopontin was positively associated with degree of liver stiffness determined using transient elastography or Fibroscan. Liver stiffness measures are well correlated with advanced stages of hepatic fibrosis and cirrhosis in adults and children[20,32,33]. These findings suggest that plasma osteopontin could serve as a potential biochemical parameter for measuring progression of liver impairment and development of hepatic fibrosis in postoperative BA patients and, therefore, may be predictive of prognosis with respect to the progression of liver dysfunction.

Several possible mechanisms could contribute to high plasma osteopontin in BA. Firstly, production of osteopontin in the damaged liver may lead to elevation of plasma osteopontin. Secondly, elevated osteopontin levels could be attributed to an imbalance between osteopontin production and osteopontin clearance. In advanced BA stages, reduced osteopontin clearance could be responsible for increased circulating osteopontin levels. Additionally, because other organs apart from the liver can synthesize and secrete osteopontin, the main sources of elevated plasma osteopontin in the present study might be extrahepatic organs. Further research will be necessary to clarify this observation.

Inevitably, we are aware of some limitations in our study. Firstly, the sample size of patients enrolled in this study was small and could preclude us from making a strong conclusion. Secondly, incomplete assessment of potential confounding factors (age, sex, medical comorbidities) needs to be taken into consideration. Lastly, as this study was designed as a cross-sectional study, a definite cause and effect relationship cannot be concluded. In order to overcome these limitations, a well-designed, well-controlled, randomized study of a large population will be needed to draw a more definite conclusion. However, with the supporting evidence from other studies regarding the potential role of osteopontin expression and the degree of systemic inflammatory response in BA[29-31], it is likely that the high plasma osteopontin levels observed in postoperative BA patients may be involved in the pathophysiology of hepatocellular injury and development of hepatic fibrosis. Biochemical parameters and clinical characteristics of some BA subjects have been reported recently[34]. We have not previously reported liver stiffness measurement or the relationship of liver stiffness with osteopontin in BA.

In conclusion, this investigation showed that BA patients had significantly elevated concentrations of plasma osteopontin in comparison with healthy controls. Plasma osteopontin and liver stiffness values were remarkably higher in the BA patients with persistent jaundice than in those without jaundice. Moreover, there was a positive correlation between plasma osteopontin, TB and liver fibrosis. Delineation of the mechanisms underlying bile duct injury will be advantageous in the development of new potential therapeutic treatments for this serious pediatric disorder.

COMMENTS

Background

Biliary atresia (BA) is an intractable liver disease of unknown cause that affects hepatic bile ducts resulting in chronic cholestasis, hepatic fibrosis and biliary cirrhosis. Human bile duct epithelial cells are able to synthesize osteopontin, a highly phosphorylated acidic glycoprotein.

Research frontiers

Osteopontin has been implicated in the pathogenesis of various inflammatory

and fibrotic disorders. However, no detailed studies are presently available on the relationship between plasma osteopontin levels and the degree of liver stiffness in BA. In this study, the authors demonstrate that elevated plasma osteopontin levels could be associated with the severity of clinical outcomes and the liver stiffness in BA patients.

Innovations and breakthroughs

Recent reports have highlighted the importance of hepatic osteopontin expression in chronic liver disease, including BA. This is the first study to report that plasma osteopontin is elevated in BA patients compared with healthy controls, and that osteopontin concentration is associated with status of jaundice and hepatic dysfunction in BA.

Applications

High plasma osteopontin levels positively correlate with the degree of hepatic fibrosis in postoperative BA children. Therefore, plasma osteopontin may be used as a biochemical parameter reflecting disease severity and for monitoring the progression of liver fibrosis in BA patients after Kasai operation.

Terminology

Osteopontin, also known as early T-cell activation gene-1, is a secreted phosphoprotein 1 that comprises multiple functional domains, with a high sialic acid content, an aspartate-rich domain, calcium-binding domain, thrombin cleavage site, and many residues with consensus for phosphorylation as well as an integrin-binding arginine-glycine-aspartate motif, which play a key role in several inflammatory disorders.

Peer review

Nice article about BA. I am unsure what the mechanism of the osteopontin levels are, but further research should surely tell us.

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BRIEF ARTICLE

Evaluation of non-ampullary duodenal polyps: Comparison of non-neoplastic and neoplastic lesions

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Abstract

AIM: To evaluate duodenal polyps, divided into non-neoplastic and neoplastic lesions. In addition, the clinical characteristics of duodenal hyperplastic polyps are determined.

METHODS: We analyzed medical records of 50 114 consecutive patients submitted to for first diagnostic esophago-gastroduodenoscopy between January 2004 and December 2009. We excluded lesions on the ampulla of Vater and submucosal tumors. We studied 510 cases that were diagnosed endoscopically with duodenal polyps and enrolled a total of 221 cases that had undergone tissue biopsy. We analyzed the differences between non-neoplastic and neoplastic lesions, and determined the clinical features of duodenal hyperplastic polyps.

RESULTS: Non-neoplastic lesions were found in 196 patients and neoplastic lesions in 25 patients. On univariate analysis, there were significant differences in shape, location, and size. Polyps more than 10 mm in diameter or polyps in the second portion had independent risk factors for being neoplastic lesions, as identified by multivariate analysis. In 23 cases of hyperplastic polyps (79.3%), they were accompanied by gastroduodenal pathology, which was possibly associated with *Helicobacter pylori*.

CONCLUSION: Polyps of more than 10 mm or polyps in the second portion of the duodenum should be evaluated by histological examination.

Key words: Duodenum; Polyp; *Helicobacter pylori*

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INTRODUCTION

The prevalence of duodenal polyps is estimated to be 0.3%-1.5% in patients referred for upper endoscopy, on the basis of extensive retrospective studies[1,2], and 4.6% reported from a prospective study[3]. Although duodenal polyps are rare, the diagnosis appears to be increasing, possibly due to the wide use of diagnostic esophago-gastroduodenoscopy. Duodenal polyps are commonly sessile,

but may also be pedunculated in nature. Most duodenal polyps are inflammatory polyps and have ectopic gastric mucosa[1,2]. Multiple and small polyps in the duodenal bulb are always benign and need neither biopsy nor treatment; except in patients with familial polyposis, where routine upper gastrointestinal surveillance endoscopy and biopsy of the duodenal polyps are mandatory[3]. Cases requiring clinical treatment are rare; however, a biopsy occasionally shows an adenoma or carcinoid tumor. These lesions require treatment due to their potential of malignant transformation. Until now, the clinical findings associated with non-neoplastic and neoplastic duodenal lesions have not been well described.

Hyperplastic (metaplastic) polyps have rarely been described in the duodenum, and published data on these lesions are limited to case reports or small case series[4-6]. They almost always occur in the setting of ectopic gastric mucosa and therefore have an appearance that more closely mimics hyperplastic polyps of the gastric type, rather than the colonic type. Moreover, when hyperplastic polyps are found in the duodenum, they can be associated with colonization by *Helicobacter pylori* (*H. pylori*)[7], and appear to occur most commonly in the setting of peptic ulcer disease or with other gastric disorders[5].

This study is the largest single-center study to date that reports on the clinical characteristics of non-ampullary duodenal elevated lesions. We aimed to evaluate the frequency, endoscopic findings, and histological characteristics of non-neoplastic and neoplastic lesions. We also analyzed a spectrum of patients with duodenal hyperplastic polyps.

MATERIALS AND METHODS

Study population

We searched the medical records of 50 114 consecutive patients that had a first diagnostic esophago-gastroduodenoscopy between January 2004 and December 2009. Most of patients had visited the gastrointestinal department and had dyspeptic symptoms. We used the electronic database of St. Vincent's Hospital, the Catholic University of Korea and searched the keywords of "duodenal polyp" (K317; International Statistical Classification of Disease, the 10th Revision, ICD-10) or "benign neoplasm of duodenum" (D132). Cases were accrued by searching the database. Among 510 cases diagnosed endoscopically with duodenal polyps, 221 cases that underwent tissue biopsy were enrolled. Lesions of the ampulla of Vater (D135) and submucosal tumors (D372) - cysts, lipomas, lymphangiectasia, and gastrointestinal stromal tumors, were excluded and were traditionally recognized as submucosal lesions (Table 1). Endoscopic and histological features of the ampullary tumors and submucosal tumors are shown in Figure 1. This study protocol was approved by the Ethics Committee of the Catholic University of Korea.

Endoscopy

Data collected on the study subjects included age, gender, family history of polyposis, and location, size, and histology of the duodenal polyp. All tissue specimens were obtained using biopsy forceps (FB-19K-1, Olympus, Tokyo, Japan), polypectomy, or endoscopic mucosal resection. The size of the polyp was measured endoscopically using the "open biopsy forceps method". Biopsy forceps with a diameter of 4 mm when fully opened were used. The forceps were withdrawn in the open position toward the endoscope tip as far as possible, until both cups were fully visualized. The open forceps was then advanced until they were aligned against the largest diameter of the polyp, with the tip of the endoscope still placed at approximately 3 to 4 cm from the polyp[8].

Table 1 Lesions in the ampulla of vater and submucosal lesions of the duodenum

Histologic diagnosis	No. (n = 44)
Lesions in the ampulla of vater	
Adenoma	12
Focal carcinomatous change	1
Inflammatory myofibroblastic tumor	1
Carcinoid	1
Submucosal lesions	
Lipoma	14
Cyst	9
Lymphangioma	3
Gastrointestinal stromal tumor	2

Study design

The endoscopic, histological, and clinical characteristics of the duodenal polyps were recorded; subsequently, the duodenal polyps were divided into non-neoplastic and neoplastic lesions. The non-neoplastic lesions included Brunner's gland hyperplasia, Brunner's gland hamartoma, ectopic gastric mucosa, ectopic pancreas, hyperplastic polyps, and inflammatory polyps. The neoplastic lesions included adenomas, carcinoid tumors, and miscellaneous lesions. We analyzed the differences of age, sex, size, shape, location and multiplicity between the non-neoplastic and neoplastic lesions. In addition, we evaluated the clinical features of the duodenal hyperplastic polyps. Each case was classified as *H. pylori* positive or negative according to the histological results (CLO test or silver stain) using two pieces of biopsy specimen taken from the antrum and the body of the stomach.

Statistical analysis

All data were recorded on standard forms and computer analyzed. The Mann-Whitney *U*-test was used to compare the continuous variables between the two groups. Differences between dichotomous variables were evaluated with the Pearson Chi-square test. Calculations were performed with the SPSS package software (SPSS version 12.0, Chicago, IL, USA). P values less than 0.05 were considered significant.

RESULTS

Demographic features

The frequency of duodenal polyps was 1.02% (510 of

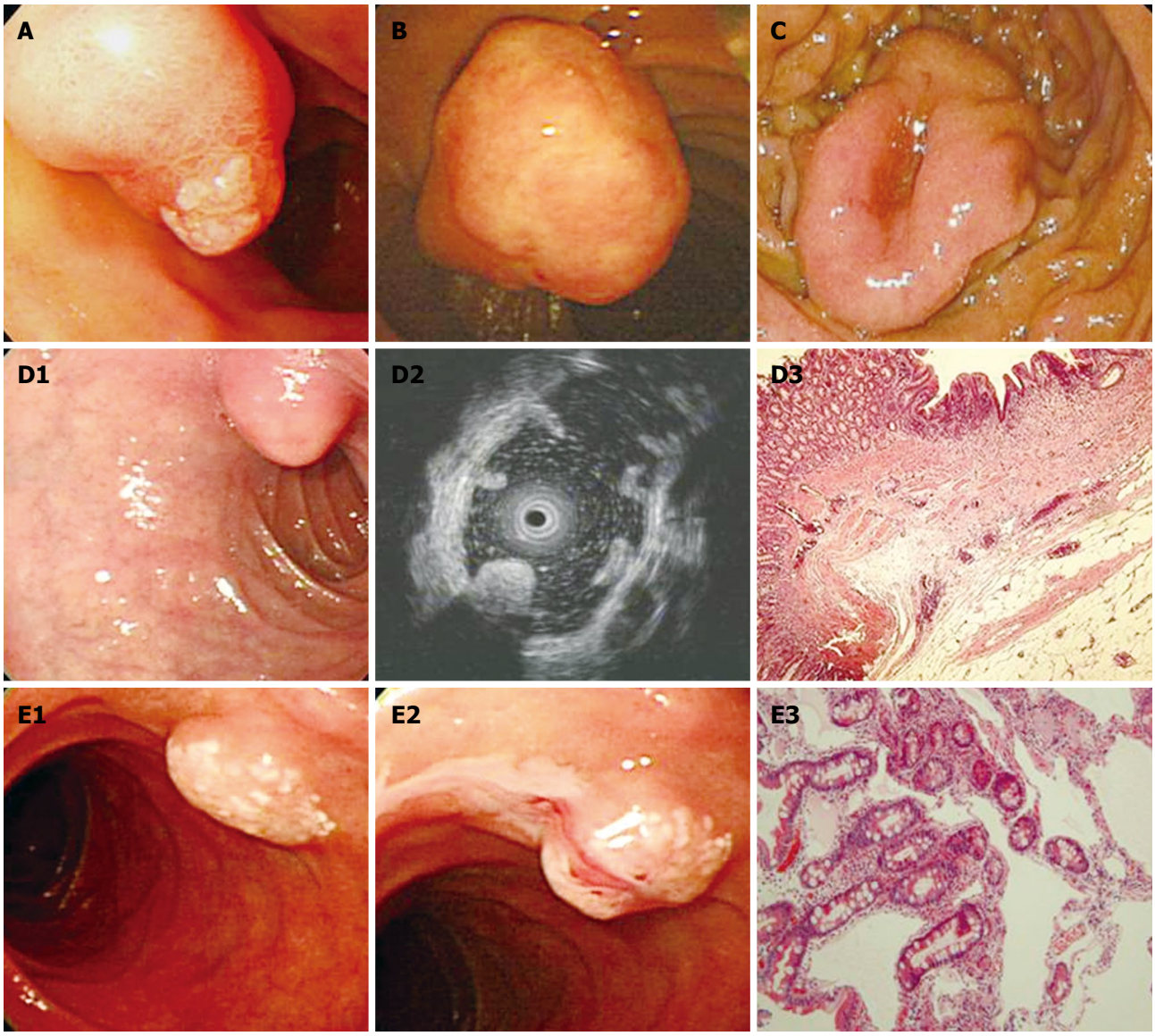

Figure 1 Ampullary polyps and submucosal lesions in the duodenum. A: Ampullary adenoma; B: Inflammatory myofibroblastic tumor; C: Gastrointestinal stromal tumor; D: Duodenal lipoma. The surface of the tumor was covered by a normal mucosa, but it had a yellowish coloration (D1). Endoscopic ultrasound showed a homogeneous and hyperechoic mass with post-acoustic shadowing (D2). Histopathological examination revealed the tumor that was composed of mature adipose tissue (HE, × 40) (D3); E: Duodenal lymphangiectasia. On the surface of the tumor, focal small whitish macules or nodules were observed (E1). After forceps biopsy, whitish milk-like material flowed out (E2). A dilated lymphatic duct in the subepithelial area was observed under microscopic examination (HE, × 40) (E3).

50114 patients). Among 221 patients, forceps tissue biopsy, and endoscopic or surgical resections were performed and a histological diagnosis was obtained. We divided them into non-neoplastic and neoplastic lesions. Non-neoplastic lesions were found in 196 patients and neoplastic lesions in 25 patients. For the non-neoplastic lesions, Brunner's gland hyperplasia/hamartoma, ectopic gastric mucosa, ectopic pancreas, hyperplastic polyps and inflammatory polyps were found. Adenomas, carcinoid tumors, and metastatic cancer were found in neoplastic lesions (Table 2). The mean age and gender ratio were not significantly different between patients having non-neoplastic and neoplastic lesions.

Endoscopic and histological features

The characteristics of the non-neoplastic and neoplastic lesions are shown in Table 2. The size of the neoplastic lesions was larger than the non-neoplastic lesions ($P < 0.01$). Non-neoplastic duodenal polyps were more frequently localized in the bulb (156/196, 79.6%) than in the second portion. In the bulb, approximately 90% of duodenal polyps were non-neoplastic. Neoplastic duodenal polyps had a tendency to be located in the second portion. Twelve (48%) out of 25 neoplastic lesions were encountered, whereas 42 (21.4%) out of 196 non-neoplastic lesions were noted ($P < 0.01$). In the bulb, neoplastic lesions comprised six cases of carcinoid tumor and seven cases of adenoma. The endoscopic findings for duodenal carcinoid tumors revealed a smooth and round elevation with an erythematous change or central depression. In one case of carcinoid tumor, a pedunculated type polyp was observed. Except for carcinoid tumors, only one case of adenoma was smaller than 10 mm in diameter. In the

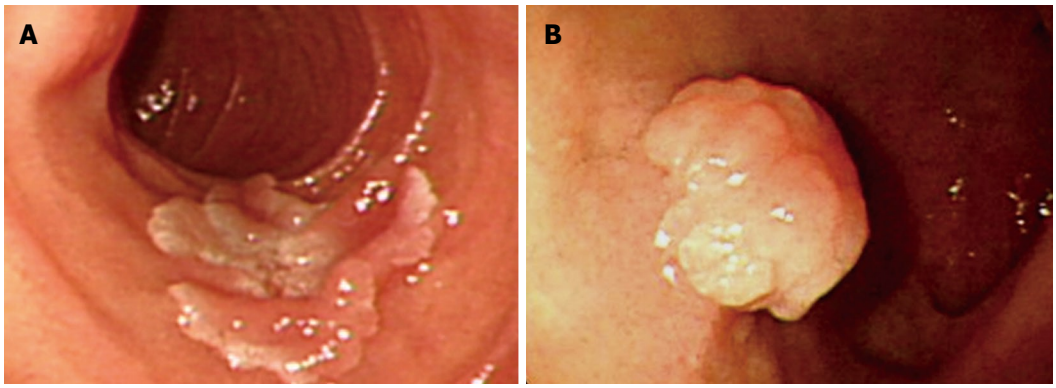

Figure 2 Endoscopic findings of duodenal adenomas. Geographic shaped flat elevated lesions (A) or semi-pedunculated polyps (B) were found in the duodenum.

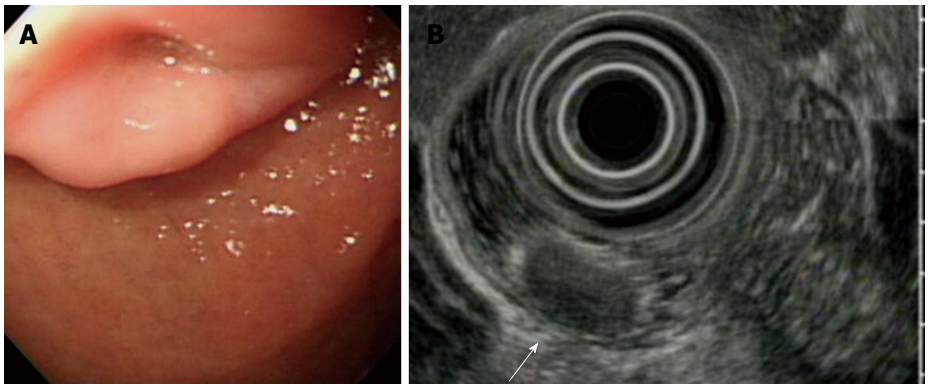

Figure 3 Endoscopic findings of carcinoid tumors. A: Endoscopy revealed an elevated lesion with a central dimpling in the bulb of the duodenum; B: Endoscopic ultrasound finding showed a round-shaped homogenous hypoechoic mass in the mucosal and muscularis mucosal layer (arrow).

Table 2 Non-neoplastic lesions and neoplastic lesions

Non-neoplastic lesions	No. (n = 196)	Neoplastic lesions	No. (n = 25)
Brunner's gland hyperplasia	17	Adenoma	14
Brunner's gland hamartoma	4	Serrated adenoma	1
		Focal cancer change in adenoma	1
Ectopic gastric mucosa	12	Originated in Brunner's gland	1
Ectopic pancreas	1	In familial adenomatosis polyposis	1
Hyperplastic polyp	29	Carcinoid tumor	6
Inflammatory polyp	33		
Normal pathology	100	Metastatic cancer	1

Table 3 Characteristics of non-neoplastic and neoplastic lesions

	Non-neoplastic (n = 196)	Neoplastic lesions (n = 25)	P value
Sex			0.66[1]
Male	122	17	
Female	74	8	
Age (yr)	56.17 ± 12.70	60.04 ± 11.24	0.14[2]
Shape			< 0.01[1]
Sessile	170	16	
Pedunculated	26	9	
Location			< 0.01[1]
Bulb	156	13	
2nd portion	40	12	
Size (cm)	0.65 ± 0.42	1.46 ± 0.76	< 0.01[2]
Multiplicity	26	2	0.71[3]

[1]Calculated by Pearson χ^2 test; [2]Calculated by Mann-Whitney U-test; [3]Calculated by Fisher exact test.

second portion of the duodenum, most of the neoplastic lesions were adenomas.

With regard to polyp shape, 9 (36%) of the 25 patients had neoplastic lesions with a semi-pedunculated or pedunculated appearance, whereas 25 (12.8%) patients had non-neoplastic lesions of similar shape (P < 0.01). Regarding the number of polyps in a single subject; 26 patients had multiple non-neoplastic lesions and only two patients had multiple neoplastic lesions. On multivariate analysis, lesions of more than 10 mm or located in the second portion of the duodenum had independent risk factors for being neoplastic lesions (Table 3). The endoscopic and histological features of the duodenal polyps are shown in Figures 2-5.

Therapeutic resection of duodenal polyps

For patients with neoplastic lesions, 20 (80%) of 25 patients had therapeutic removal and 16 (64%) patients had

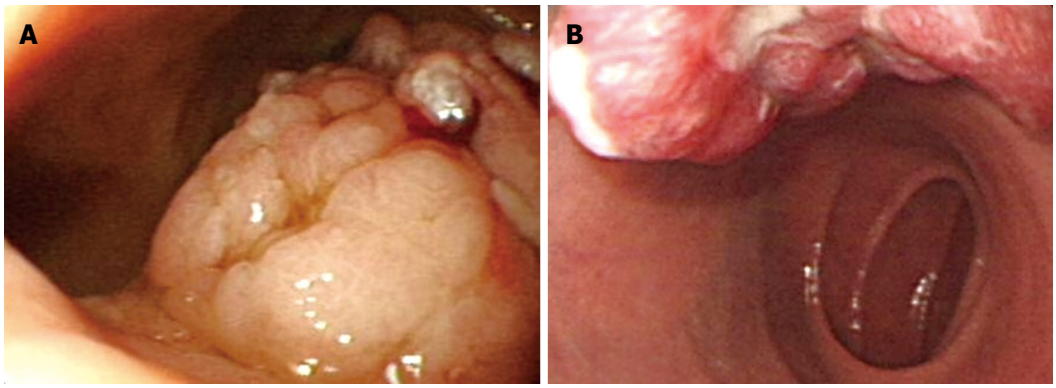

Figure 4 Serrated adenoma (A) and focal cancer change of adenomas (B). Large pedunculated polyps were observed in the duodenal bulb. They had multilobulated and friable surfaces.

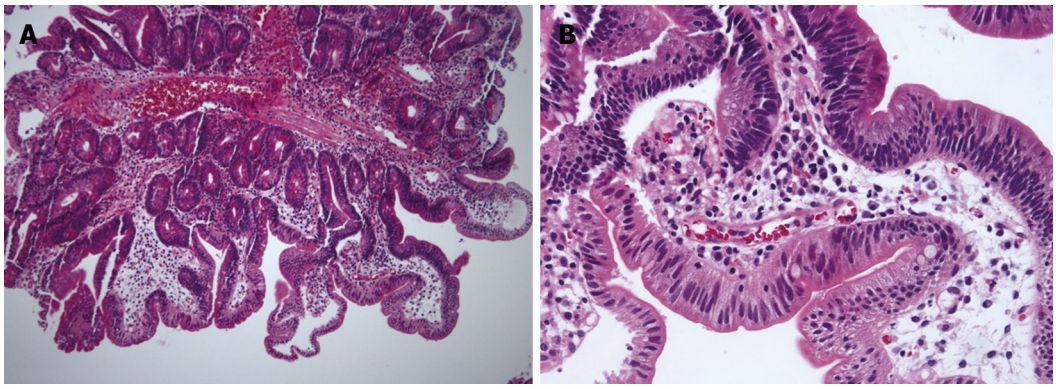

Figure 5 Microscopic finding of serrated adenomas. A: Vascular stalk and saw-tooth appearance were observed (HE, × 40); B: At high magnification, hyperplastic foveolar cells were found. In part, epithelia with pleomorphic, stratified nuclei and irregular chromatin deposits were observed (HE, × 200).

Table 4 Independent risk factors identified on multivariate analysis

Total	Odds ratio	95% CI	*P* value
Type	1.56	0.54-4.46	0.41
Sessile			
peduncle			
Location	3.55	1.32-9.54	0.01
Bulb			
2nd portion			
Size (cm)	14.12	4.99-39.99	< 0.01
< 1.0			
⩾ 1.0			

Table 5 Frequencies of gastroduodenal lesions associated with duodenal hyperplastic polyps

	n (%)
Peptic ulcer	24.1 (7)
Gastric cancer	3.4 (1)
Chronic atrophic gastritis	58.6 (17)
Gastroesophageal reflux disease	10.3 (3)
Gastric hyperplastic polyp	6.8 (2)
Miscellaneous[1]	6.8 (2)

[1]Gastric varix, gastric submucosal tumor.

successful endoscopic resection. Two of the remaining five patients have chosen surveillance endoscopy, because the lesion was small and the patients had co-morbid diseases. Two cases were lost to follow-up. One patient with a metastatic lesion from esophageal cancer has received radiation therapy.

Duodenal hyperplastic polyps

Hyperplastic polyps were detected in 29 patients. Two cases had adenomatous changes or focal cancer changes with an adenomatous component. Gastroesophageal lesions identified with duodenal hyperplastic polyps are shown in Table 4. In 21 patients, the *H. pylori* status was evaluated according to the histological results and 12 (57.1%) patients had *H. pylori* infection. The clinical features accompanying duodenal hyperplastic polyps are shown in Table 5. Twenty-three patients (79.3%) were had associated gastroduodenal pathology; peptic ulcer, gastric cancer, gastric hyperplastic polyp, and chronic atrophic gastritis, which was possibly associated with *H. pylori* infection.

DISCUSSION

In our retrospective study, the prevalence of incidentally found duodenal polyps was 1.02% among cases undergo-

ing routine diagnostic esophago-gastroduodenoscopy. The prevalence confirmed by histology was 0.44%. These results were consistent with the frequencies disclosed in previous reports. However, there were several limitations. First, the study population and the object of esophago-gastroduodenoscopy were not uniform-screening or diagnostic procedure for the gastrointestinal symptoms. Second, a tissue biopsy was not performed in all cases of duodenal polyp. Out of 510 polyps, only 221 were biopsied and the others were probably considered as non-neoplastic.

Several types of polyps can occur in the duodenum and most of them are non-neoplastic. Non-neoplastic duodenal polyps include Brunner's gland hyperplasia, ectopic gastric mucosa, and hyperplastic polyps. Previous retrospective studies showed that ectopic gastric mucosa and Brunner's gland hyperplasia are common in the duodenum, and that hyperplasic polyps were extremely rare[6,9,10]. However, in this study, most of the non-neoplastic polyps had normal duodenal mucosa or the lesions had distinguishable histopathology suggestive of an inflammatory polyp. In addition, hyperplastic polyps are a common type of duodenal polyp. Our results are similar to those of a previous prospectively designed study[3]. Inflammatory polyps are presumed to be part of a diffuse inflammatory process, with metaplastic or regenerative changes of the overlying mucosa on histology[4]. The duodenal inflammatory polyps were smaller than the other types of polyps.

Brunner's gland hyperplasia and hamartomas were often encountered in the bulb of the duodenum[11,12], and our results showed that 76.2% (16/21 cases) were located in the bulb. The pathogenesis of these lesions is mostly unknown, and might be associated with duodenal injury observed in patients with acid hypersecretion. Macroscopically, they tend to be solitary sessile or pedunculated polyps with a central pore and are located in the posterior wall of the duodenal bulb. Their histological features include the proliferation of a mixture of mesenchymal tissues, such as adipose, smooth muscle, and Brunner's gland, and cystic dilation of the Brunner's gland. Rarely, adenoma or carcinoma can develop from a Brunner's gland and in symptomatic cases with bleeding, endoscopic removal is necessary[13,14]. In our data, three cases were treated with a polypectomy for Brunner's gland hyperplasia/hamartoma; in one case an adenoma developed from a Brunner's gland and two cases were large polyps.

Although most duodenal polyps had a benign clinical course, some of them can show malignant transformation into adenomas or carcinoid tumors. Therefore, early diagnosis and treatment are important for these cases. In this study, adenomas were the most frequently detected neoplastic lesions, consistent with the findings of previous studies[6,9,10,15]. One case of an adenoma developed from a Brunner's gland, and another case was associated with familial adenomatous polyposis (FAP). In addition, an adenoma of mixed hyperplastic and adenomatous morphology was observed as a serrated adenoma. A serrated adenoma of the duodenum is a very uncommon lesion and has been described in association with FAP[16]. A sporadic case of serrated adenoma has not been previously reported in the duodenum, and this appears to be the first report of one in the literature.

The results of this study showed that the neoplastic lesions were larger than the non-neoplastic lesions, and had a significant predilection for the second portion of the duodenum compared with the non-neoplastic lesions. In addition, a previous report suggested that multiple, small polyps in the duodenal bulb are always benign and need neither biopsy nor treatment. Moreover, polyps in the second portion of the duodenum are rare. If polyps were present in the second portion of the duodenum, a substantial number would be adenomas[17]. Although duodenal carcinoid tumors were rare, their detection and treatment are important. On endoscopic examination, they were yellowish colored and had central dimpling or ulceration with erythematous changes[18]. It should be kept in mind that a polyp-like lesion might be a carcinoid tumor. For the subset of lesions measuring between 1 and 2 cm in diameter, endoscopic ultrasound (EUS) may ultimately offer the best diagnostic method to help guide the management of individual patients[19]. Nearly all neoplastic lesions were treated by local resection (e.g.: polypectomy or mucosal resection) or surgery, except for a metastasis from an esophageal cancer. Most of them could be managed with endoscopic therapy with minimal morbidity. If the lesions located in the second portion of the duodenum cannot be excised by endoscopic therapy, a pancreatico-duodenectomy may be required. However, this technique is associated with a high complication rate.

There have been several reports of hyperplastic gastric polyps associated with persistent *H. pylori* gastritis[20-22], but the association of *H. pylori* with duodenal hyperplastic polyps has been rarely described. Two case reports suggested that these lesions are rare and associated with *H. pylori* infection[6,7]. As a result, their clinical features are poorly understood. Based on our results, most of the patients with duodenal hyperplastic polyps had inflammatory mucosal pathology of gastric mucosa: chronic atrophic gastritis, peptic ulcer, or gastric cancer. A large number of patients were infected with *H. pylori*. Moreover, the association of *H. pylori* might have been underestimated because of the retrospective study design, and the fact that we could not exclude past exposure to *H. pylori*. Unfortunately, there was no control group and the interpretation of this result is limited.

In conclusion, although the frequency of clinically significant duodenal polyps was low, the polyps of more than 10 mm or polyps in the second portion of the duodenum should be carefully observed and evaluated by histological examination. Duodenal hyperplastic polyps might have an association with *H. pylori* infection and with various forms of gastro-duodenal disease.

COMMENTS

Background

Duodenal polyps are rare, although the diagnosis appears to be increasing, possibly due to the wide use of diagnostic endoscopy. Several types of polyps can oc-

cur in the duodenum. However, the clinical importance of non-ampullary duodenal polyps is not well understood and clear management guidelines do not exist.

Research frontiers

The clinical and endoscopic characteristics of neoplastic non-ampullary duodenal polyps are not well known; therefore, this study evaluated their features, including age, sex, size, and endoscopic characteristics (location and gross appearance). In addition, the clinical characteristics of duodenal hyperplastic polyps were determined.

Innovations and breakthroughs

On univariate analysis, there were significant differences in shape, location,and size. Polyps of more than 10 mm in diameter or polyps in the second portion had independent risk factors for being neoplastic lesions identified on multivariate analysis. 79.3% of the hyperplastic polyps were accompanied by gastroduodenal pathology that was possibly associated with *H. pylori* infection.

Applications

This study provides a consensus on guidelines for clinical management of non-ampullary duodenal polyps and helps to discriminate neoplastic polyps.

Peer review

The study reports a retrospective single center evaluation of the prevalence and histology of duodenal polyps in over 50 000 subjects undertaking upper G-I endoscopy for several reasons. The main finding of the authors, on multivariate analysis, is that polyps of the 2° duodenal portion have a greater risk of being neoplastic. The study is interesting because of the large database analyzed; however the retrospective and hospital-based modality of screening should be better acknowledged as a limitation.

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BRIEF ARTICLE

Brain activity following esophageal acid infusion using positron emission tomography

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Abstract

AIM: To investigate symptoms and brain activity following esophageal acid infusion.

METHODS: Fifteen healthy volunteers were recruited for the study. Hydrochloric acid (pH 1 and 2) and distilled water (pH 7) were randomly and repeatedly infused into the esophagus. The brain activity was evaluated by positron emission tomography. The severity of heartburn elicited by the infusion was rated on an auditory analog scale of 0-10.

RESULTS: The severity of heartburn following each infusion showed a step-wise increase with increasing acidity of the perfusate. The heartburn scores were significantly higher in the second pH 1 infusion compared with the first infusion. Acid and distilled water infusion induced activation of various brain areas such as the anterior insula, temporal gyrus, and anterior/posterior cingulate cortex. At pH 1 or 2, in particular, activation was observed in some emotion-related brain areas such as the more anterior part of the anterior cingulate cortex, parahippocampal gyrus, or the temporal pole. Strong activation of the orbitofrontal cortex was found by subtraction analysis of the two second pH 1 infusions, with a significant increase of heartburn symptoms.

CONCLUSION: Emotion-related brain areas were activated by esophageal acid stimulation. The orbitofrontal area might be involved in symptom processing, with esophageal sensitization induced by repeated acid stimulation.

Key words: Esophageal acid infusion; Brain imaging; Positron emission tomography

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INTRODUCTION

Gastroesophageal reflux disease (GERD) causes reflux symptoms such as heartburn and regurgitation due to reflux of the gastric contents into the esophagus, with or without mucosal damage[1]. Although there is a correlation between the severity of esophagitis and acid reflux, it is known that the severity of subjective symptoms is not necessarily correlated with that of acid reflux[2]. In particular, heartburn symptoms are weakly correlated with acid reflux in non-erosive reflux disease (NERD)[3], and NERD patients are often resistant to treatment with acid-suppressive medication[4]. Therefore, the possible involvement of esophageal hypersensitivity in NERD patients has attracted attention[5].

It has been shown that NERD patients show hypersensitivity not only to mechanical stimulation, but also to acid and/or non-acid chemical stimulation[6-8]. Some investigators have argued that the susceptibility of afferent nerve terminals to luminal acid based on the dilated intercellular space in the esophageal mucosa is important as a causative factor for acid hypersensitivity[9,10]. Recent studies have demonstrated that weak acid or gas reflux is associated with the generation of reflux symptoms[11]. However, perceived acid reflux accounts for only a minority of reflux events[12], and the mechanism of heartburn symptoms remains to be elucidated[13].

Recently, brain imaging analysis using positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) has been employed in visceral sensation studies as an objective evaluation tool for the processing mechanism of perception[14]. This advanced approach has demonstrated that some important brain areas, such as the anterior cingulate cortex (ACC) or insula, are involved in the processing of visceral sensation and pain, and abnormality or modulation of these brain areas in patients with irritable bowel syndrome[15-17]. Until now, these brain imaging studies of the viscera, especially of the rectum or colon, have been mainly conducted using barostat-controlled methods; a mechanically extended stimulation device[18]. Only a few studies have investigated brain activity after esophageal chemical stimulation, such as hydrochloric acid[19-21]. The aim of this study was to investigate induced symptoms and brain activity using PET in esophageal acid stimulation.

MATERIALS AND METHODS

Subjects

Fifteen right-handed healthy adult male volunteers (mean age: 26.7 years; range: 21-37 years), who had no typical reflux symptoms such as heartburn and regurgitation, were recruited for the study that was conducted from October 2005 to June 2007. All subjects were healthy volunteers with no gastrointestinal disorders and signs. It was confirmed that they had no prior history of craniocerebral trauma or intracranial diseases.

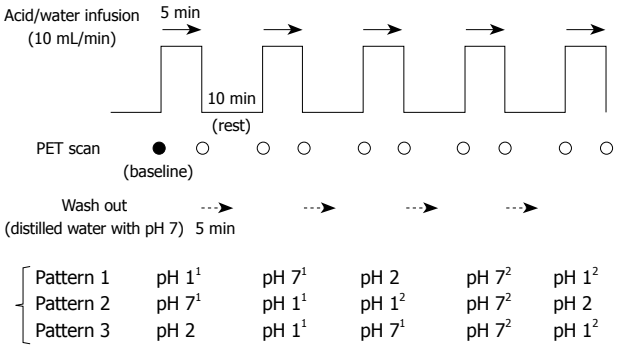

Figure 1 This schema illustrates the procedure of esophageal infusion and brain positron emission tomography scanning. The infusions were performed twice for pH 1 and 7 solutions (distilled water) and once for the pH 2 solution. In order to counterbalance the effects of the infusion order, the order was randomly selected per each subject from pH 1-7-2-7-1, pH 7-1-1-7-2 and pH 2-1-7-7-1 as shown. [1]First infusion; [2]Second infusion; PET: Positron emission tomography.

A small-diameter catheter (new enteral feeding tube 3393-5; Nippon Sherwood Medical Industries Ltd., Tokyo, Japan) was inserted transnasally into the esophagus, and fixed at 35 cm from the exterior nostril. A wired pH glass electrode (CM-181; Chemical Appliance Co. Ltd., Tokyo, Japan), pre-attached at its proximal side 3 cm from the infusion catheter, was connected to a pH meter. The placement of the catheter and pH electrode in the middle esophagus was confirmed by chest X-ray. Subjects were placed in a supine position in the PET scanner with their heads immobilized in a head immobilization device to control head movement during scanning. In order to obtain correction data for γ-ray absorption in the body, subjects initially underwent a transmission scan using a ^{68}Ge/^{68}Ga radiation source. This study was approved by the Ethics Committee for Human Research at Tohoku University Graduate School of Medicine, Sendai, Japan. Informed consent was obtained from every subject.

Esophageal acid infusion

The procedures for esophageal infusion and PET scan are schematically shown in Figure 1. Infusions of 50 mL HCl (pH 1 and 2) or distilled water (pH 7) were provided by a catheter using an automatic syringe pump (Terufusion syringe pump TE-312; Terumo Co. Ltd., Tokyo, Japan) for a total of 5 min at 10 mL/min. The infusions were performed twice for pH 1 and pH 7 solutions and once for the pH 2 solution. In order to counterbalance the effects of the infusion order, the order was randomly selected from pH 1-7-2-7-1, pH 2-1-7-7-1, and pH 7-1-1-7-2. Then ^{15}O-labeled water was administered intravenously in synchronization with the completion of each 5-min infusion. After confirming that the brain activity could be detected, a PET emission scan of the head was performed for 60 s prior to the PET scan; the subjects were instructed to remain awake during the scan that was performed in a darkened room. Using a PET scanner (Headtome-V set- 2400 W; Shimadzu, Kyoto, Japan)[22] in a 3D data acquisition mode, a total of 10 scans were taken

before and after each of the five infusions, to measure the regional cerebral blood flow in each subject. After each acid infusion, the esophagus was neutralized by an infusion of distilled water (pH 7) over 5 min at 10 mL/min and by additional swallowing. During 10-min intervals between infusions, it was confirmed that the esophageal pH was 4 or higher, as an indicator of the non-acidic status[23], and that the radioactivity in the heads of the subjects had returned to baseline (pre-scan) levels. Subjects were asked to rate the severity of heartburn symptoms on an analog scale of 0-10 after each infusion, and the resultant scores were used in the analysis of data. The incidence of heartburn symptoms and the heartburn scores elicited after the five infusions for each of the two groups were statistically analyzed by Fisher's test and the Wilcoxon signed-ranks test, respectively. Differences were considered statistically significant when the P value was < 0.05.

PET data analysis

The PET data were transferred to a super computer (NEC, SX-4/128H4, Tohoku University Cyberscience Center, Sendai, Japan) and PET images were reconstructed using a 3D filtered back projection algorithm[24]. Realignment, spatial normalization, and smoothing of images were performed using statistical parametric mapping (SPM) software (SPM 2, Wellcome Department of Cognitive Neurology, London, UK), and significantly different changes in regional cerebral blood flow were mapped. All regional cerebral blood flow images were anatomically normalized against a standard brain space such as the Montreal Neurological Institute atlas[25]. The standardized images were smoothed using a 12 mm × 12 mm × 12 mm Gaussian filter. Evaluations of regional cerebral blood flow were adjusted using analysis of covariance and mean scaling set at 50, and expressed in mL/min per 100 g. The effects of grouping and co-variability were each evaluated using a general linear model of voxels.

The following two analyses were performed to determine the areas of regional brain activity that correlated with the esophageal acid infusion. First, brain images taken following infusion with hydrochloric acid (pH 1 and 2) or distilled water (pH 7), as well as images taken at baseline (prior to all infusions) were subjected to subtraction analysis to investigate the brain regions that were activated by each infusion. Next, the effects of repeated infusion of acid or distilled water were assessed by subtraction analysis of images obtained following the first and second infusions with pH 1 and pH 7 solutions. All statistical methods were evaluated using linear convolution and contrasts, and the voxel values for each image were constructed using a statistical parametric map of the t-statistic statistical parametric mapping. The location of statistical peaks was determined in Talairach and Tournoux atlas. P (uncorrected) < 0.001 was defined as statistically significant for increased cerebral blood flow.

RESULTS

Enhanced incidence and severity of symptoms following acid infusion

The incidence of heartburn symptoms following each infusion was 33.3% for the first pH 7 infusion, 33.3% for the second pH 7 infusion, 46.7% for pH 2, 66.7% for the first pH 1 infusion, and 80.0% for the second pH 1 infusion. The incidence of heartburn symptoms following each infusion showed a step-wise increase with increasing acidity of the perfusate. The incidence of heartburn tended to be higher after the second pH 1 infusion than after the first, and these scores were significantly increased following the second pH 1 infusion. On the other hand, the heartburn incidence and scores in both pH 7 infusions were much lower compared to the pH 1 infusions. Symptom scores were significantly increased after the pH 2 infusion compared to the second pH 7 infusion, and after the second pH 1 infusion compared to the pH 2 infusion (Table 1).

Table 1 Incidence of heartburn symptoms and heartburn scores induced by each infusion

	Heartburn incidence	Mean heartburn scores (range)
pH 7 (first infusion)	5/15	1.4 (0-7)
pH 7 (second infusion)	5/15	1.0 (0-6)
pH 2	7/15	1.9 (0-9)[b]
pH 1 (first infusion)	10/15	3.2 (0-10)[c]
pH 1 (second infusion)	12/15[a]	5.0 (0-10)[d]

[a]$P = 0.0253$ *vs* pH 7 (first infusion) and pH 7 (second infusion); [b]$P = 0.0269$ *vs* pH 7 (second infusion); [c]$P = 0.0464$ *vs* pH 7 (first infusion), $P = 0.0253$ *vs* pH 7 (second infusion); [d]$P = 0.0040$ *vs* pH 1 (first infusion), $P = 0.0075$ *vs* pH 2, $P = 0.0041$ *vs* pH 7 (first infusion), $P = 0.0071$ *vs* pH 7 (second infusion).

Activated brain areas following acid infusion

Comparison of brain images following each infusion: The brain image obtained at rest prior to all infusions was defined as the baseline image. Differences between brain images at baseline and those taken after infusion with acid or distilled water were subjected to subtraction analysis. Brain regions with increased blood flow were defined as those neurologically activated by each infusion. The details of the brain regions activated following each infusion are shown in Table 2 and are summarized in Table 3.

After the first pH 7 infusion, activation was observed in the right precentral gyrus, left superior temporal gyrus, right and left ACC, right anterior insula and, after the second pH 7 infusion, in the right middle frontal gyrus, cuneus (center), right posterior cingulate cortex (PCC), right postcentral gyrus, right ACC, left inferior frontal gyrus, left middle temporal gyrus, right and left thalamus, and right superior temporal gyrus. The regions activated at pH 2 were the left cerebellum, right inferior frontal gyrus, left superior temporal gyrus (temporal pole, BA38), right anterior insula, left putamen, left PCC, right ACC, mid pons, and left superior temporal gyrus. After the first pH 1 infusion, activation was observed in the right precentral gyrus, right superior temporal gyrus (temporal pole, BA38), left middle temporal gyrus, left parahippocampal gyrus, left ACC, and left middle temporal gyrus. After the second pH 1 infusion, activation was observed in the right parahippocampal gyrus (Figure 2A), left superior temporal gyrus (temporal

Table 2 Details of brain activated regions by acid infusion (comparison with baseline)

Condition	Region	Side	BA	x	y	z	Z-score	Voxels in cluster
Frist pH 7 - base	Precentral gyrus	Right	4	56	-8	22	4.23	252
	Superior temporal gyrus	Left	42	-64	-12	6	3.80	72
	Anterior cingulate cortex	Left	24	-12	6	34	3.78	34
	Anterior cingulate cortex	Right	24	12	4	28	3.59	17
	Anterior insula	Right		30	-4	20	3.43	15
Second pH 7 - base	Middle frontal gyrus	Right	10	36	44	-4	4.51	79
	Cuneus	Center		0	-104	-2	4.02	45
	Posterior cingulate cortex	Right	23	28	-52	10	3.84	58
	Postcentral gyrus	Right	1,2	44	-20	32	3.57	19
	Anterior cingulate cortex	Right	24	12	4	26	3.49	31
	Inferior frontal gyrus	Left	47	-50	34	-2	3.49	21
	Middle temporal gyrus	Left	21	-64	-4	-22	3.45	18
	Thalamus	Left		-20	-36	4	3.44	41
	Thalamus	Right		18	16	16	3.44	26
	Superior frontal gyrus	Right	10	10	66	24	3.21	10
pH 2 - base	Cerebellum	Left		-2	-72	-20	4.01	59
	Inferior frontal gyrus	Right	45	34	10	20	3.94	40
	Superior temporal gyrus	Left	38	-52	20	-24	3.85	18
	Anterior insula	Right		30	-4	22	3.78	68
	Putamen	Left		-22	-12	10	3.60	35
	Posterior cingulate cortex	Left	31	-26	-62	12	3.36	28
	Anterior cingulate cortex	Right	24	6	32	-2	3.35	15
	Pons	Center		0	-26	-30	3.34	12
	Superior frontal gyrus	Left	10	-20	52	-8	3.34	14
First pH 1 - base	Precentral gyrus	Right	6	68	4	20	3.85	36
	Superior temporal gyrus	Right	38	32	10	-38	3.80	39
	Middle temporal gyrus	Left	21	-44	-30	-10	3.74	24
	Parahippocampal gyrus	Left		-26	-50	6	3.74	46
	Anterior cingulate cortex	Left	24	-12	26	-2	3.67	20
	Middle temporal gyrus	Left	21	-64	-2	-22	3.48	22
Second pH 1 - base	Parahippocampal gyrus	Right		28	-48	2	4.47	129
	Superior temporal gyrus	Left	38	-52	18	-26	4.12	24
	Cerebellum	Left		-20	-40	-50	4.09	58
	Posterior cingulate cortex	Left	23	-8	-20	26	3.93	15
	Caudate nucleus	Left		-14	-26	20	3.70	32
	Anterior insula	Right		36	12	-14	3.63	27
	Pons	Right		12	-42	-32	3.43	57
	Caudate nucleus	Left		-24	-38	12	3.24	16

Table 3 Summary of brain activated regions by each infusion (comparison with baseline)

Major brain region	Subregion	BA	First pH 7	Second pH 7	pH 2	First pH 1	Second pH 1
Frontal lobe	Superior frontal gyrus	10		R	L		
	Middle frontal gyrus	10		R			
	Inferior frontal gyrus	47		L			
Temporal lobe	Superior temporal gyrus	38			L	R	L
	Superior temporal gyrus	42	L				
	Middle temporal gyrus	21		L		L	
	Middle temporal gyrus	42	L		L		
	Inferior temporal gyrus	45			R		
PMA	Precentral gyrus	4	R				
	Precentral gyrus	6				R	
PSA	Postcentral gyrus	1,2,3		R			
ACC	Anterior part	24			R	L + R	
	Mid/posterior part	24′	L + R	R			
PCC				R	L		L
Insula	Anterior part		R		R		R
Cerebellum					C + L		L
Thalamus				R + L			R + L
PHG						L	R

PMA: Primary motor area; PSA: Primary somatosensory area; ACC: Anterior cingulate cortex; PCC: Posterior cingulate cortex; PHG: Parahippocampal gyrus; BA: Brodmann area; R: Right; L: Left; C: Center.

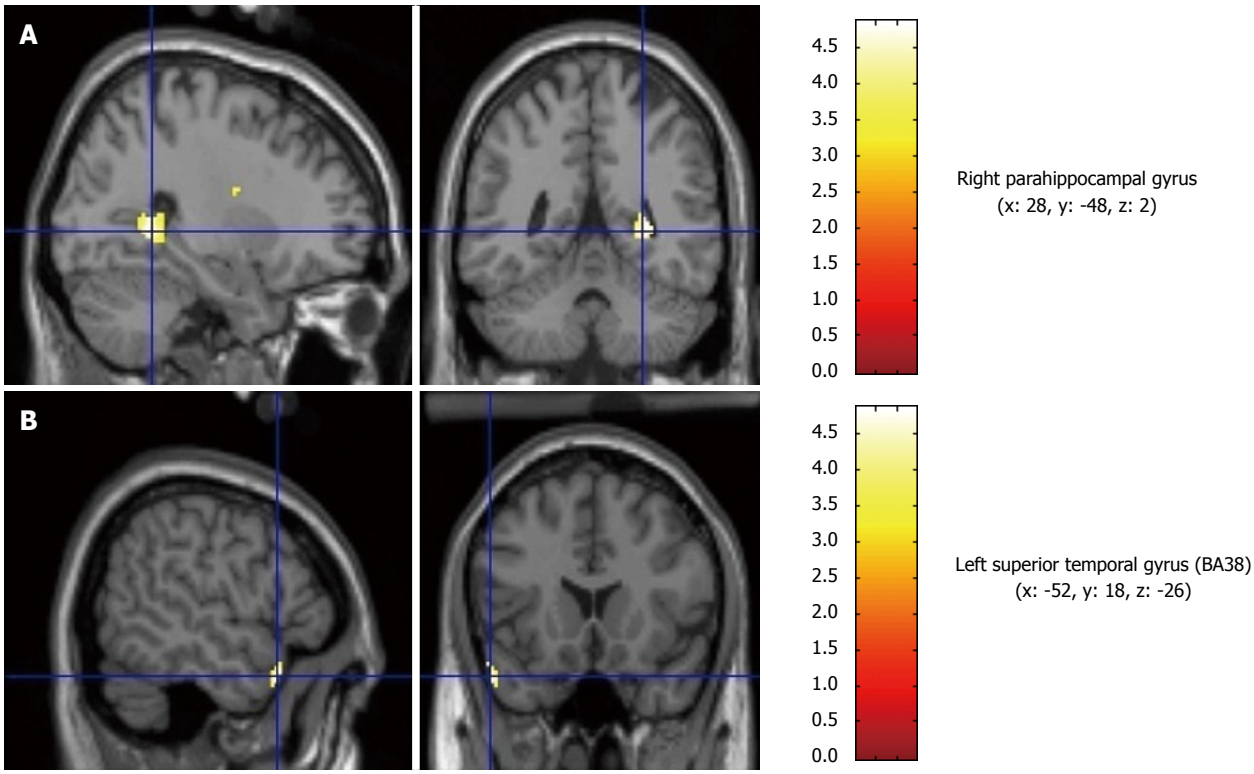

Figure 2 Representative images from the subtraction analysis of the second pH 1 infusion minus the baseline. Left: Sagittal view; Right: Cranial view. A: Right parahippocampal gyrus (x: 28, y: -48, z: 2); B: Left superior temporal gyrus (temporal pole, BA38) (x: -52, y: 18, z: -26).

pole, BA38) (Figure 2B), left cerebellum, left PCC, left caudate nucleus, right anterior insula and right pons.

In view of the acidity level of the perfusate, brain activation was observed in the prefrontal area at pH 2 and 7 but not at pH 1. In the insula, activation was observed at the second pH 1 and 2 and first pH 7 infusions. Activation in the cingulate cortex was observed in nearly all infusions, with no particular trend observed for the topography of the activated sub-regions. At pH 1 and 2, activation was observed in the more anterior (rostral) part of the ACC (BA 24a) and, at pH 7, in the more posterior (dorsal) part of the ACC (BA 24a'). Many activated areas were observed in regions of the temporal gyrus, with no particular trend observed for the topography. After infusions at pH 1 and 2 but not pH 7, activation was observed in the temporal pole (BA 38). Activation was also observed in the cerebellum following infusions at pH 1 and 2, and in the parahippocampal gyrus after both pH 1 infusions. On the other hand, the frontal area, precentral gyrus, and thalamus were less activated after each infusion.

Comparison of brain imaging with first and second infusion at pH 1 and 7: As described above, the scores for heartburn symptoms after the pH 1 infusions were significantly increased after the second infusion compared to the first. When we analyzed the difference of these two conditions using subtraction analysis, the second pH 1 infusion minus the first showed that cerebral blood flow was increased in the right orbitofrontal cortex (Figure 3A), right cuneus, left cerebellum, right superior temporal gyrus, right middle frontal gyrus (Figure 3B), right pons, right lingual gyrus, left putamen, and right caudate nucleus. On the other hand, the result of the second pH 7 infusion minus the first showed an increase in cerebral blood flow in the right middle frontal gyrus, left cerebellum, right midbrain, left PCC, and right superior frontal gyrus. Those brain areas are summarized in Table 4.

DISCUSSION

In the present study, we found that brain activity was substantially increased in the cingulate cortex and frontal lobe following esophageal acid infusion, with little activity observed in the thalamus and somatosensory areas. The insula was not consistently activated by acidic or non-acidic stimulations in this study, but in previous studies, the activation of the insula and ACC has been highly reproducible, and is considered to play a central role in the integration of visceral sensation[14,17]. ACC is functionally and anatomically divided into several subregions[26]. The more anterior part of the ACC (BA 32, 25, 24) is involved in affective and emotional responses and the dorsal part (BA 32', 24') is involved in cognitive processes[27]. Activation of the ACC predominantly occurred in the anterior part (BA 24) at pH 1 and 2, and in the posterior part (BA 24') at pH 7 in this study, which supports the fact that heartburn is uncomfortable and troublesome[1].

The heartburn symptom scores following infusions at pH 1 and 2 were higher compared with those at pH 7. We found that the parahippocampal gyrus was activated only by pH 1 infusion. This area is an important part of the limbic system, which plays a major role in the processing

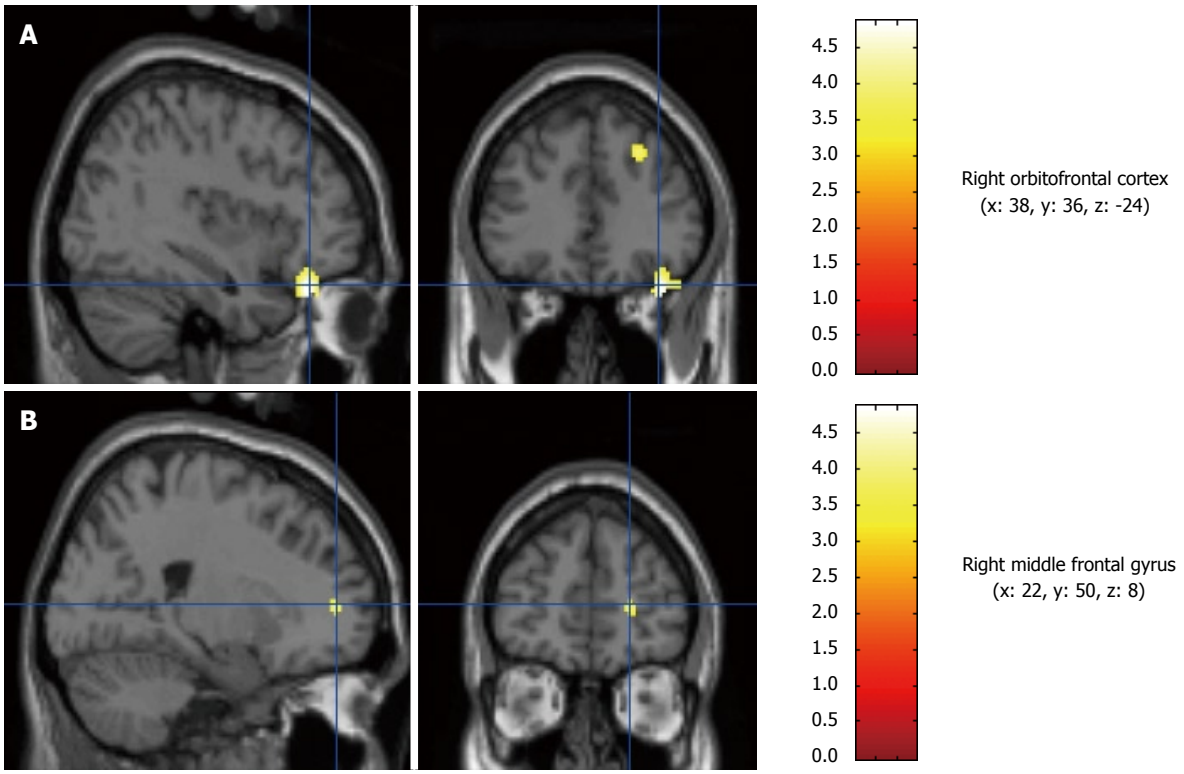

Figure 3 Representative images from the subtraction analysis of the second pH 1 infusion minus the first. Left: Sagittal view; Right: Cranial view. A: Right orbitofrontal cortex (x: 38, y: 36, z: -24); B: Right middle frontal gyrus (x: 22, y: 50, z: 8).

Table 4 Results of subtraction analysis of brain images after the first and second infusions at pH 1 and 7

Condition	Region	Side	Brodmann area	x	y	z	Z-score	Voxels in cluster
Second pH 1 - first	Orbitofrontal cortex	Right		38	36	-24	4.44	167
	Cuneus	Right	19	2	-82	36	3.79	51
	Cerebellum	Left		-22	-40	-50	3.74	23
	Superior temporal gyrus	Right	8	26	36	40	3.69	37
	Middle frontal gyrus	Right	32	22	50	8	3.69	23
	Pons	Right		10	-36	-12	3.47	31
	Lingual gyrus	Right	19	4	-60	-2	3.37	12
	Putamen	Left		-24	-8	2	3.32	16
	Caudate nucleus	Right		18	-34	16	3.30	16
Second pH 7 - first	Middle frontal gyrus	Right	10	36	46	0	4.03	47
	Cerebellum	Left		-18	-98	-18	3.98	40
	Midbrain	Right		2	-40	2	3.65	27
	Posterior cingulate cortex	Left	23	-14	-16	30	3.61	11
	Superior frontal gyrus	Right	8	14	22	42	3.57	33

of emotional reaction or memory[28]. Therefore, activation of the parahippocampal gyrus is compatible with induction of uncomfortable heartburn by acid infusion. Also, activation was observed in the temporal pole following infusions at pH 1 and 2, but not at pH 7. The temporal pole (BA 38) is located at the anterior extremity of the temporal lobe, which includes the superior and middle temporal gyri. There have been few reports regarding activation of the temporal pole in previous studies of esophageal sensation. However, a recent study has shown that this area is activated by distention in the proximal stomach[29], and another report has described activation of the temporal pole by distention of the descending colon, with a feeling of anxiety[30]. In a study using photographs as visual stimulation, the temporal pole was activated by emotions of comfort and discomfort, wakefulness, and attended stimulation[31]. Therefore, the activation of the temporal pole observed in our study could have been due to alterations in the level of arousal, attention and emotion following acid infusion. These observations also suggest that, as shown by the activation pattern of the ACC in this study, high-acidity stimulation in the esophagus can induce emotional responses.

It has been shown that heartburn is apt to be perceived when preceding acid reflux or prior acid stimulation exists[11,32,33]. Also in this study, the heartburn scores were significantly higher after the second pH 1 infusion compared to the first, which suggests that esophageal sensation was

sensitized by repeated acid infusion. These changes were not observed after repeated infusion at pH 7. Visceral sensitization, which can occur at the primary afferent nerve level (peripheral sensitization) and/or the spinal cord level (central sensitization), is considered as a very important phenomenon in the development of visceral sensation[34]. Finally, visceral sensation is perceived through intracerebral processing and modulation[34]. Recent studies using cortical evoked potentials or fMRI have reported that esophageal sensitization induced by acid stimulation results in alterations in the neural activity of the ACC and insula[35-37]. In our present study, subtraction analysis of the second pH 1 minus the first showed that increased brain activity occurred in several areas, including the right orbitofrontal cortex, right supratemporal gyrus and right middle frontal gyrus. Of those brain areas, the orbitofrontal cortex had the highest Z-score and cluster level in our study. The orbitofrontal cortex, which is frequently observed to be activated following stimulation of the lower gastrointestinal tract, was less activated following esophageal stimulation in previous studies[14,38,39]. As a higher center of sensory integration, this area is thought to participate in the assessment of reward, punishment, comfort, discomfort, and memory or its verification[40]. Thus, our findings showed that the orbitofrontal cortex, besides the ACC or insula, shown in other studies[35-37], might also play a role in symptom processing with esophageal acid sensitization.

Chemoreceptor stimulation of the esophagus is also thought to activate fine sympathetic and parasympathetic afferents. Fine sympathetic afferents ascend the lamina I of the spinal cord, and parasympathetic afferents provide input to the solitary tract nucleus[28]. These activities are integrated in the parabrachial nucleus, which projects to the posterior dorsal insula by way of (or by passing) the ventromedial thalamic nuclei[28]. In humans, this cortical image is represented in the anterior insula[28]. This is compatible with our findings documenting the activation of the anterior part whenever the insula is activated, although its activation was not detected under condition with the second pH 1 infusion minus the first. However, these representations provide the foundation for a subjective evaluation of the interoceptive state, which is forwarded to the orbitofrontal cortex, where hedonic valence is represented[40], and was depicted as discrimination between the first and the second acidic stimuli in the present study.

In the present study, little activation was observed in the thalamus and primary somatosensory cortex. Based on previous reports of visceral and somatic sensation, the activation in the primary somatosensory cortex has poorer reproducibility compared with activation in the insula; possibly due to variations in the intensity, property, and spatial and temporal amount of stimulation in the respective studies[14,17,41]. Especially in visceral sensation, the total amount of spatial and temporal stimulation is very difficult to evaluate, which could account for the variation in brain activation. Alternatively, the lower activation in the primary somatosensory cortex suggests vague localization of pain originating from the viscera[17]. Moreover, in esophageal acid infusion tests, including our present and previous studies, it is necessary to take into account the differences in the acidity, infusion rate, total volume of the perfusate, and the position during infusion, which probably influence the induction of symptoms and concomitant brain activation[20,21,42,43]. Kern *et al*[21] have reported in an fMRI study that activation in the sensory motor cortex among GERD patients with luminal/perceived esophageal acid exposure was substantially higher than that in healthy controls with subliminal acid stimulation. This study suggests that the sensory motor area is associated with the perception of heartburn symptom in GERD patients, although it does not apply to healthy controls. A further study of our patients with GERD is needed.

In the present study, pH 1 and 2 hydrochloric acid was chosen as an acidic stimulant of the esophagus. pH 1 hydrochloric acid has been traditionally and widely used as an esophageal chemical stimulant[6,20,21,32,33,44], and there is a report that pH 2 represents a critical level of acidity in inducing heartburn symptoms[45]. Due to the time constraints of our PET facility, infusions at pH 2 were performed only once, whereas infusion at pH 1 and 7 was performed twice. This could be a limitation of our study. We also randomly selected one of the three aforementioned infusion orders for each subject to counterbalance the influence of the infusion order. The infusion order was not revealed to the subjects, but anticipation of the infusion might have influenced the brain activation[46]. On the other hand, the physical and mental stress associated with keeping still for a long period, the gag reflex and swallowing with an indwelling intranasal tube might have an adverse effect on brain activation[43].

In summary, this present study using PET showed that the insula, cingulated gyrus, temporal gyrus, and cerebellum were activated in esophageal acid perception in healthy volunteers, and that involvement of the somatosensory and prefrontal areas was minimal. In particular, emotion-related brain regions such as the anterior part of ACC, the parahippocampal gyrus and the temporal pole were activated under acidic conditions in the esophagus. It is also suggested that activation of the orbitofrontal area is involved in esophageal sensitization to repeated acid stimulation at the cerebral level. Dysfunction of these brain areas may be associated with the pathogenesis of functional heartburn or non-erosive reflux disease. Further studies of brain imaging to elucidate the mechanism of esophageal acid perception and sensitization in healthy subjects and patients with GERD, including NERD and functional heartburn, are warranted.

COMMENTS

Background

Esophageal hypersensitivity is a potentially causative factor in the pathogenesis of non-erosive reflux disease (NERD) or functional heartburn. In those patients, there may also be a neural alteration at the brain level against esophageal acid reflux, but little is documented on this issue.

Research frontiers

Using brain positron emission tomography (PET), we sought to analyze the symptoms and brain activity following esophageal acid infusion with different pH levels in healthy volunteers.

Innovations and breakthroughs

Several emotion-related brain areas such as the anterior part of the anterior cingulate cortex, parahippocampal gyrus or the temporal pole were activated by esophageal acid stimulation. In addition, a strong activation of the orbitofrontal cortex was observed with repeated pH 1 HCl perfusions, with a significant increase of heartburn symptoms.

Applications

This preliminary study might contribute to the authors' understanding of the pathogenesis of NERD or functional heartburn, and could provide a newly therapeutic agent that targets an alteration in brain activity induced by acid reflux.

Peer review

The authors investigated the symptoms and brain activity following esophageal acid infusion, by PET and auditory analog scale in healthy volunteers. They found that emotion-related areas were activated under conditions of high acidity, with heartburn symptoms, and that the orbitofrontal area might be involved in symptom processing, with esophageal sensitization induced by repeated acid stimulation. Their observations could contribute to an elucidation of the pathogenesis of NERD or functional heartburn, and could be helpful for development of new therapeutic options.

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BRIEF ARTICLE

Predictors of rebleeding after initial hemostasis with epinephrine injection in high-risk ulcers

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Abstract

AIM: To identify the predictors of rebleeding after initial hemostasis with epinephrine injection (EI) in patients with high-risk ulcers.

METHODS: Recent studies have revealed that endoscopic thermocoagulation, or clips alone or combined with EI are superior to EI alone to arrest ulcer bleeding. However, the reality is that EI monotherapy is still common in clinical practice. From October 2006 to April 2008, high-risk ulcer patients in whom hemorrhage was stopped after EI monotherapy were studied using clinical, laboratory and endoscopic variables. The patients were divided into 2 groups: sustained hemostasis and rebleeding.

RESULTS: A total of 175 patients (144, sustained hemostasis; 31, rebleeding) were enrolled. Univariate analysis revealed that older age (≥ 60 years), advanced American Society of Anesthesiology (ASA) status (category Ⅲ, Ⅳ and Ⅴ), shock, severe anemia (hemoglobin < 80 g/L), EI dose ≥ 12 mL and severe bleeding signs (SBS) including hematemesis or hematochezia were the factors which predicted rebleeding. However, only older age, severe anemia, high EI dose and SBS were independent predictors. Among 31 rebleeding patients, 10 (32.2%) underwent surgical hemostasis, 15 (48.4%) suffered from delayed hemostasis causing major complications and 13 (41.9%) died of these complications.

CONCLUSION: Endoscopic EI monotherapy in patients with high-risk ulcers should be avoided. Initial hemostasis with thermocoagulation, clips or additional hemostasis after EI is mandatory for such patients to ensure better hemostatic status and to prevent subsequent rebleeding, surgery, morbidity and mortality.

Key words: Epinephrine injection; High-risk ulcers; Initial hemostasis; Predictors; Rebleeding

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INTRODUCTION

Bleeding peptic ulcer is a common and life-threatening

medical emergency[1]. With regard to endoscopic hemostasis, epinephrine injection (EI) monotherapy is a common and effective endoscopic method of hemostasis because of its low risk, low cost and high accessibility[2-4]. Although EI monotherapy has good efficacy in the hemostasis of bleeding peptic ulcers, bleeding recurs in about 10%-30% of the population[5-8]. Undoubtedly, recurrent bleeding remains the most important adverse independent prognostic factor[9,10]. Recent studies have revealed that thermocoagulation, sclerosant injection or clips alone, or combined with EI are superior to EI alone for preventing rebleeding, surgery and mortality. EI alone is not recommended[11-15]. However, the reality is that EI monotherapy to arrest ulcer bleeding is still commonly practiced, in part, because it is a relatively simple and effective hemostatic method and because some patients are intolerant of time-consuming EI combination therapy[16-18]. As sustained hemostasis is the goal of endoscopic therapy, we tried to identify factors which predicted recurrent bleeding after achieving initial hemostasis with EI monotherapy. Therefore, an additional hemostatic method to EI, or initial thermocoagulation, sclerosant injection or clips is warranted in such high-risk patients to ensure better hemostatic efficacy.

MATERIALS AND METHODS

Patient selection

Based on previous validations, those ulcers with spurting bleeding (Ⅰa), oozing bleeding (Ⅰb), non-bleeding visible vessel (NBVV, Ⅱa) and adherent clots (Ⅱb) were high-risk ulcers according to the Forrest classification and should be considered for endoscopic therapy[4,19]. From October 2006 to April 2008 in the Medical Center, Chang Gung Memorial Hospital, Kaohsiung, Taiwan, we enrolled patients with high-risk ulcers (Ⅰ, Ⅱa and Ⅱb) who achieved initial hemostasis with EI monotherapy (epinephrine solution, 1:10000) into this study. Patients who failed to achieve hemostasis during EI monotherapy or received endoscopic combination therapy were excluded. Moreover, patients with malignant ulcers were also excluded. Pharmacotherapy with proton pump inhibitors (PPIs) in these patients was also recorded.

Clinical, laboratory and endoscopic assessments

We analyzed the possible factors which predicted recurrent bleeding in patients with successful initial hemostasis. The clinical, laboratory and endoscopic variables investigated included age, sex, Forrest class, ulcer size, ulcer location, hemoglobin level, blood transfusion, difficulty in the injection approach, injection dose, patient status including outpatient (in the emergency room due to ulcer bleeding) and inpatient (development of ulcer bleeding during admission) and bleeding presentation including severe bleeding signs (SBS; hematemesis or hematochezia) or mild bleeding signs (MBS; coffee ground vomitus or melena). Shock status was defined as either a systolic blood pressure of less than 90 mmHg, or less than 100 mmHg plus a pulse rate of more than 100 beats per minute. The clinical risk status of the patients was assessed by means of the American Society of Anesthesiology (ASA) classification[20]. That is, ASA Ⅰ = healthy patient, ASA Ⅱ = patient with mild systemic disease without functional limitation, ASA Ⅲ = severe systemic disease with definite functional limitation, ASA Ⅳ = severe systemic disease that is a constant threat to life, and ASA Ⅴ = moribund patient not expected to survive for more than 24 h with or without surgery. The hemoglobin level was recorded before blood transfusion and endoscopic therapy. The total amount of packed red blood cells (pRBC) transfused was recorded until endoscopic hemostasis was achieved. Use or non-use of nonsteroidal anti-inflammatory drugs (NSAID) and aspirin was also recorded. The accessibility of injection therapy was categorized into an easy or difficult approach depending on the location of the ulcers. Peptic ulcers located in the lesser curvature and posterior wall of the gastric body and posterior wall of the duodenum were regarded as those that required a difficult approach for injection therapy. Peptic ulcers located in areas other than the above-mentioned were regarded as easy approach. Rebleeding was defined as the recurrence of bleeding within 2 wk of initial hemostasis.

Statistical analysis

Univariate analysis for possible factors predicting recurrent bleeding was performed using the Pearson χ^2 test and Fisher's exact test for categorical variables and the Student's t test for continuous variables. Variables with a $P < 0.05$ were considered statistically significant. Variables with a $P < 0.4$ in the univariate analysis were used for multiple logistic regression analysis with backward stepwise correction and were considered independent predictors of recurrent bleeding with a $P < 0.05$.

RESULTS

From October 2006 to April 2008, a total of 662 sessions of EI-based procedures to treat upper gastrointestinal bleeding were recorded in our computerized medical record system. After excluding patients who underwent endoscopic combination therapy, endoscopic hemostasis failure, and those who had malignant ulcer bleeding or Mallory-Weiss tear bleeding, a total of 175 patients with high-risk ulcers (Ⅰ, Ⅱa and Ⅱb) who achieved initial hemostasis were enrolled. All these patients received intravenous PPI therapy during the acute bleeding period followed by oral PPIs to maintain hemostasis. Our records indicated that 144 patients (82.3%) achieved sustained hemostasis and 31 patients (17.7%) suffered from recurrent bleeding. Univariate analysis revealed that older age (age ≥ 60 years), advanced ASA status (category Ⅲ, Ⅳ and Ⅴ), shock, severe anemia (hemoglobin < 80 g/L), injection dose ≥ 12 mL and SBS were risk factors of recurrent bleeding (Table 1). However, backward stepwise correction (Table 2) revealed that only older age ≥ 60 years, odds ratio (OR) 5.11, 95% confidence interval (CI): 1.34, 19.48,

Table 1 Predictors of recurrent bleeding after epinephrine injection therapy in the univariate analysis

	Sustained hemostasis	Recurrent bleeding	*P* value
Patient number	144	31	
Age (yr)	63.7 (±14.4)	68.0 (±11.3)	0.035
< 60	55	5	0.019
⩾ 60	89	26	
Sex			0.375
Male	100	24	
Female	44	7	
ASA category			0.006
Ⅰ, Ⅱ	44	2	
Ⅲ, Ⅳ, Ⅴ	100	29	
Shock			0.003
Negative	119	18	
Positive	25	13	
Forrest class			0.973
Active bleeding (Ⅰa, Ⅰb)	98	21	
Recent bleeding (Ⅱa, Ⅱb)	46	10	
Ulcer size (cm)			1.000[1]
< 2	128	28	
⩾ 2	16	3	
Injection approach			0.689
Easy	80	16	
Difficult	64	15	
Ulcer location			0.512
Stomach	79	15	
Duodenum	65	16	
Pre-endoscopic blood transfusion (mL)	658 ± 475	758 ± 463	0.277
< 1000	92	19	0.785
⩾ 1000	52	12	
Hemoglobin (g/dL)	9.5 ± 2.4	6.6 ± 1.7	< 0.001
⩾ 8	104	6	< 0.001
< 8	40	25	
Injection dose (mL)	8.7 ± 4.1	10.4 ± 5.0	0.057
< 12	127	21	0.011
⩾ 12	17	10	
Bleeding signs			< 0.001
Mild	119	16	
Severe	25	15	
Patient status			0.076
Outpatient	81	12	
Inpatient	63	19	
NSAID/aspirin			0.738
User	51	10	
Non-user	93	21	

[1]The value was obtained according to Fisher's exact test based on an expectation value < 5. NASID: Nonsteroidal anti-inflammatory drug.

hemoglobin < 80 g/L (OR 13.44, 95% CI: 4.29, 42.13), injection dose ⩾ 12 mL (OR 5.72, 95% CI: 1.69, 19.38) and SBS (OR 5.46, 95% CI: 1.89, 15.79) were independent predictors. All 31 rebleeding patients received repeated endoscopic therapies, and only 14 of them achieved permanent hemostasis. The rest suffered from recurrent bleeding and 10 underwent surgery. In summary, among the 31 patients who re-bleeded after initial endoscopic hemostasis with EI alone, 15 (18/31, 48.4%) encountered delayed hemostasis causing major complications such as sepsis, hypovolemic shock, and renal and respiratory failure, and 13 (13/31, 41.9%) died of these complications (Table 3). The overall clinical course of the 175 patients is listed in Figure 1.

Table 2 Predictors of recurrent bleeding after epinephrine injection therapy from stepwise logistic regression in the multivariate analysis

	Odds ratio	95% CI	*P* value
Age (yr)			
⩾ 60 *vs* < 60	5.11	1.34-19.48	0.017
Hemoglobin (g/dL)			
< 8 *vs* ⩾ 8	13.44	4.29-42.13	< 0.001
Bleeding signs			
Severe *vs* mild	5.46	1.89-15.79	0.002
Injection dose (mL)			
⩾ 12 *vs* < 12	5.72	1.69-19.38	0.005

Variables of $P < 0.4$ were used in the multivariate analysis. CI: Confidence interval.

Table 3 The outcome of 31 patients with rebleeding after initial hemostasis using epinephrine injection therapy

Outcome of rebleeding patients	No. of patients (n = 31)
Surgery	10 (32.2%)
Major complications[1]	15 (48.4%)
Sepsis	8
Renal failure	2
Respiratory failure	8
Hypovolemic shock	2
Death	13 (41.9%)

[1]Some patients suffered 2 or more major complications related to recurrent bleeding.

DISCUSSION

Due to medical progress in the management of ulcer hemorrhage, pharmacotherapy with PPIs and endoscopic hemostasis are standard treatments. PPIs are the first choice of pharmacotherapy to control ulcer bleeding at present due to their strong inhibition of acid secretion and promotion of platelet aggregation[21]. PPIs have also been demonstrated to reduce rebleeding, the need for surgery and repeated endoscopic therapy[22]. With regard to endoscopic therapy, this treatment also reduces the occurrence of rebleeding, the need for surgery and the morbidity and mortality of patients. When bleeding recurs, however, repeated endoscopic therapy may either prevent patients from undergoing surgery or delay surgical hemostasis[23,24]. Therefore, the most important goal of endoscopic therapy is to initially achieve permanent hemostasis. Recently, numerous meta-analyses have indicated that adding a second procedure, such as a second injectate (alcohol, thrombin, sclerosant or fibrin glue), thermocoagulation or clips to EI significantly reduced rebleeding, surgery and mortality compared with EI alone in high-risk ulcer patients[11-15]. EI alone is not recommended in the management of bleeding ulcers; however, the reality is that endoscopic hemostasis with EI alone is still commonly practiced[16-18]. There are several reasons for this: first, injection monotherapy is a simple and effective hemostatic method (82.3% in the current study). Second, EI combination therapy is

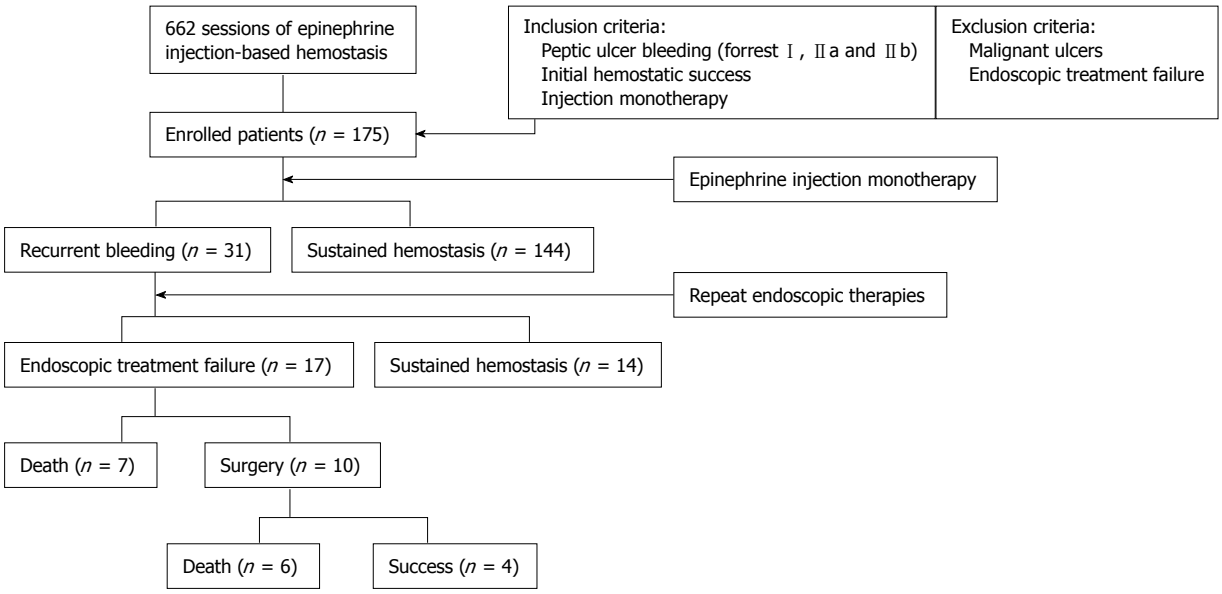

Figure 1 The clinical course of 175 patients with successful initial hemostasis after endoscopic epinephrine injection monotherapy.

a relatively time-consuming procedure, so patients may not tolerate or complete the process, particularly those in a hemodynamically unstable status. Third, thermotherapy such as argon plasma coagulation (APC) or mechanical hemostasis with clips may not be available in every endoscopic unit, particularly in local hospitals. Therefore, to identify predictors of recurrent bleeding in high-risk ulcer patients after EI alone may justify the benefit of endoscopic combination therapy or initial replacement by thermocoagulation or clips for a better treatment outcome. Those patients with such risk factors treated with EI only should be closely monitored or referred to other hospitals with a well-equipped endoscopic unit. From the high-risk ulcer patients treated by PPIs pharmacotherapy and EI alone in our study, we found older age (≥ 60 years), advanced ASA categories (Ⅲ, Ⅳ and Ⅴ), shock, severe anemia (hemoglobin < 80 g/L), injection dose ≥ 12 mL and SBS were the factors which predicted rebleeding. Older age, severe anemia, high injection dose and SBS were independent predictors in multivariate analysis.

Compared to ulcers with NBVV or black spots, active ulcer bleeding tends to rebleed if left untreated. After successful endoscopic hemostasis and administration of PPIs, there were no statistical differences observed in this study. Severe anemia and SBS were relevant to the amount of blood loss and the severity of ongoing bleeding, and were risk factors for recurrent bleeding. The amount of blood transfused before endoscopic therapy may not correlate well with the severity of blood loss. In our hospital, a 24-h emergency endoscopy service is provided and patients often undergo early therapeutic endoscopy within 24 h of either visiting the emergency room or the occurrence of bleeding after admission. Thus, the amount transfused prior to early endoscopy cannot be considered as a risk factor of rebleeding. As for the use of large and small volumes of EI in bleeding ulcers, there are two prospective studies which refer to the hemostatic effectiveness of injection volume. In these studies, the injection of a larger volume might reduce the rate of recurrent bleeding compared to a smaller volume in a prospective design[25,26]. On the other hand, the need for a higher injection dose to arrest bleeding might imply a difficult hemostasis and a higher risk of recurrent bleeding from a retrospective viewpoint. In our study, an injection dose ≥ 12 mL was an independent risk factor of recurrent bleeding.

NSAID and aspirin also cause peptic ulcer bleeding. Prior use of NSAID/aspirin was reported to increase the risk of rebleeding in bleeding ulcer patients[27,28]. If the use of NSAID is discontinued, PPI therapy is very effective in treating NSAID-related ulcers and preventing further bleeding.

It is well known that *Helicobacter pylori* (*H. pylori*) infection can cause peptic ulcers. The detection of *H. pylori* was not performed in our study as these patients were in an acute bleeding phase. The rapid urease test during the acute bleeding phase is unreliable for the detection of *H. pylori* infection[29]. Furthermore, Schilling *et al*[30] also revealed that *H. pylori* infection does not affect the early rebleeding rate in patients with peptic ulcer bleeding after successful endoscopic hemostasis, however, patients should be tested and treated for *H. pylori* infection once their condition has stabilized to prevent recurrent ulcers.

EI monotherapy is commonly practiced in endoscopic hemostasis due to its good efficacy, and because it is a simple and time-saving technique[2-4]; however, it should be replaced by combination therapy, clips, sclerosant injection or thermocoagulation based on more recent evidence. Sclerosant injection, clips or thermocoagulation alone or in combination with EI are more effective methods than EI alone. Although we achieved sustained hemostasis in 82.3% of our patients with bleeding ulcers treated by endoscopic EI monotherapy, 31 patients suffered from recurrent bleeding. The outcome of the patients with re-

bleeding was discouraging as 15 patients (48.4%) suffered from major complications and 13 patients (41.9%) died of complications. Among the 31 rebleeding patients, 10 patients underwent surgical hemostasis but only 4 patients survived. Although these rebleeding patients often had underlying major diseases, to achieve sustained hemostasis using sclerosant, thermocoagulation, clips or EI combination as soon as possible would allow these patients to have a better prognosis. Therefore, we suggest that all patients with high-risk ulcers should undergo sclerosant injection, thermocoagulation or clips in index endoscopy because they are more effective and time-saving. EI followed by thermocoagulation, clips or another injectate should also be considered, however, these are more time-consuming and some patients cannot tolerate or complete the procedures. Unless sclerosant, APC or clips are unavailable, EI alone is not recommended, particularly in older patients (> 60 years), SBS, severe anemia, and those in need of a higher EI dose to arrest bleeding during endoscopic therapy. Additional endoscopic therapies to EI are mandatory or these patients should be referred to a hospital with a well-equipped endoscopic unit for close monitoring.

In conclusion, endoscopic EI monotherapy in patients with high-risk ulcers should be avoided. Initial hemostasis with thermocoagulation, clips or additional hemostasis after EI is mandatory, particularly in older patients, SBS, severe anemia and where a higher injection dose in need. Then, it is possible to ensure that such patients have a better hemostatic status and will avoid subsequent rebleeding, surgery, morbidity and mortality.

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COMMENTS

Background

Recent studies have revealed that thermocoagulation, sclerosant injection or clips alone, or combined with epinephrine injection (EI) are superior to EI alone in preventing rebleeding, surgery and mortality. EI alone is not recommended. However, the reality is that EI monotherapy to arrest ulcer bleeding is still common practice, in part, because it is a relatively simple and effective hemostatic method and because some patients are intolerant of time-consuming EI combination therapy.

Research frontiers

Sustained hemostasis is the goal of endoscopic therapy. This study identified the factors which predicted rebleeding if patients with high-risk ulcers were treated using EI alone to achieve initial hemostatic status.

Innovations and breakthroughs

Endoscopic EI monotherapy in patients with high-risk ulcers should be avoided. Initial hemostasis with thermocoagulation, clips or additional hemostasis after EI is mandatory, particularly in older patients, SBS, severe anemia, and where a higher injection dose is needed to prevent subsequent rebleeding, surgery, morbidity and mortality.

Applications

This article further emphasized the need to follow the existing guidelines for the treatment of high-risk ulcers to possibly avoid a poor outcome when EI is performed alone.

Terminology

High-risk ulcers refer to ulcers with active bleeding, or with non-bleeding visible vessels or adherent clots, and endoscopic therapy is necessary in these ulcers to prevent further bleeding.

Peer review

The clinical study focused on identification of predictors of rebleeding after initial epinephrine hemostasis in the upper gastrointestinal tract. The statistical approach revealed age, severe bleeding signs, hemoglobin, and epinephrine injection dose more than 12 mL as predictors.

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BRIEF ARTICLE

Gastric juice acidity in upper gastrointestinal diseases

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Abstract

AIM: To search the independent factors determining gastric juice acidity and to investigate the acidity of gastric juices in various benign and malignant upper gastrointestinal diseases.

METHODS: Fasting gastric juice acidity of 165 healthy subjects and 346 patients with esophageal ulcer (n = 21), gastric ulcer (n = 136), duodenal ulcer (n = 100) or gastric cancer (n = 89) were measured and compared. Additionally, gastric specimens were taken from the antrum and body for rapid urease test and histological examination.

RESULTS: Multivariate analysis revealed that bile stain of gastric juice, high acute inflammatory score of the corpus, and atrophy of the corpus were independent risk factors for the development of gastric hypoacidity with odds ratios of 3.1 (95% CI: 1.3-7.3), 3.1 (95% CI: 1.2-7.9) and 3.5 (95% CI: 1.3-9.2). Esophageal ulcer and duodenal ulcer patients had a lower pH level (1.9 and 2.1 *vs* 2.9, both $P < 0.05$) of gastric juices than healthy subjects. In contrast, gastric ulcer and gastric cancer patients had a higher pH level (3.4 and 6.6 *vs* 2.9, both $P < 0.001$) than healthy controls. Hypoacidity existed in 22%, 5%, 29%, 5% and 88% of healthy subjects, esophageal ulcer, gastric ulcer, duodenal ulcer and gastric cancer patients, respectively.

CONCLUSION: Bile reflux, atrophy and dense neutrophil infiltrate of the corpus are three independent factors determining the acidity of gastric juice.

Key words: Acidity; Gastric juice; Gastric cancer; Peptic ulcer; Esophageal ulcer

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INTRODUCTION

Gastric juices are liquids found in the stomach. They contain numerous compounds, including hydrochloric acid (HCl), pepsin, lipase, mucin, intrinsic factor, peptides, nucleic acids and electrolytes[1]. Additionally, they may also contain salivary constituents due to swallowing, bile due to gastroduodenal reflux and inflammatory mediators or blood from damaged gastric walls[2]. In their normal state, gastric juices are usually clear in color.

HCl is an important component in gastric juice. It is a strong acid produced by the parietal cells in the corpus generating a gastric pH of 2-3[1]. Activation of pepsin and absorption of nutrients relies on an acidic pH in the stomach. HCl is also important in protecting the stomach and intestines from pathogens. Increased gastric pH induced by disease process, reflux of bile or pharmaceuticals allows for bacterial overgrowth in the stomach[3]. These pathogenic bacteria in the hypochlorhydria stomach can produce nitrite and nitroso-compounds, which act as one of the triggers in the atrophy-metaplasia-dysplasia-carcinoma pathway[4].

Helicobacter pylori (*H. pylori*) infection is an important biological factor which can induce marked alterations in gastric acid secretion of hosts[5,6]. In subjects with an antrum-predominant gastritis following *H. pylori* infection there is increased release of gastrin and consequently increased acid secretion. Such subjects have an increased risk of developing duodenal ulcers (DU)[7,8]. In contrast, the infection induces a corpus-predominant gastritis with hyposecretion of acid in some subjects. These infected subjects have an increased risk of developing gastric cancer (GC)[9,10]. Gastric juices can lead to mucosal damage when they enter the esophagus. Patients with gastroesophageal reflux diseases (GERD) may develop esophageal breaks, along with damage to the enamel of the teeth caused by the high acidity of the stomach contents[11].

Since the acidity of gastric juice is one of the crucial factors in the development of most upper gastrointestinal diseases, we designed this study to search the independent factors determining gastric juice acidity and to investigate the acidity of gastric juices in various upper gastrointestinal diseases.

MATERIALS AND METHODS

Subjects

One hundred and sixty-five consecutive healthy subjects (HSs), 21 patients with esophageal ulcer (EU), 136 patients with gastric ulcer (GU), 100 patients with DU and 89 patients with GC participated in the study. The HSs recruited from our health examination clinics had no clinical history of gastrointestinal diseases, and their endoscopic findings were normal or showed mild gastritis only. The diagnoses of EU, GU and DU were confirmed by endoscopic examination. An EU was defined as a well-defined mucosal break present in the lower esophagus[12]. A gastric or duodenal ulcer was defined as a circumscribed mucosal break 5 mm or more in diameter, with a well-defined ulcer crater in the stomach or duodenum, respectively[13]. The size of ulceration was measured by opening a pair of biopsy forceps of known span in front of the ulcer. The diagnosis of GC was confirmed by histology. Additionally, GC was classified as intestinal (n = 50), diffuse (n = 31) and mixed (n = 8) according to the Lauren's classification[14]. The patient exclusion criteria included (1) the use of proton pump inhibitors or H2-receptor antagonists within 4 wk prior to the study; (2) coexistence of two kinds of gastroduodenal lesions; (3) presentation with upper gastroduodenal bleeding; and (4) coexistence of severe systemic diseases. The study was approved by the Medical Research Committee of the Kaohsiung Veterans General Hospital. All patients and controls gave informed consent.

Clinical methods

Endoscopies were performed with the Olympus GIF XV10 and GIF XQ200 (Olympus Co., Tokyo, Japan) after patients had fasted overnight. Immediately after insertion of the scope into the stomach, 5 mL of gastric fluid was aspirated through the suction channel of the endoscope and collected in a sterile trap placed in the suction line for acidity assay and color assessment. Routine inspection of the upper gastrointestinal tract was then performed. Additionally, gastric specimens were taken for rapid urease test (one specimen from the antrum) and histological examination (two specimens from the antrum and another two from the body)[15].

To adjust for clinical characteristics, the following data were recorded for each subject: age, sex, family history of gastric cancer, smoking, alcohol drinking, coffee consumption, tea consumption.

Acidity and color of gastric juice

The pH of gastric juice was measured just after collection with a glass-electrode pH meter. Hypoacidity was defined as pH level of gastric juice greater than 3.5[16]. The color of gastric juice was carefully assessed, and bile stain of gastric juice was defined as yellowish or greenish discoloration of the gastric juices.

Rapid urease test

The rapid urease test was performed according to our previous studies[17]. Each biopsy specimen was placed immediately in 1 mL of a 10% solution of urea in deionized water (pH 6.8) to which two drops of 1% phenol red solution had been added and incubated at 37℃ for up to 24 h. If the yellowish color around the area of inserted specimen changed to bright pink within the 24-h limit, the urease test was considered positive. In our laboratory, the sensitivity and specificity of the rapid urease test were 96% and 91%, respectively.

Histological assessment

A histological examination of the stomach was carried out for the subjects who provided informed consent for topographical histopathological study. The biopsy specimens were fixed in 10% buffered formalin, embedded in

Table 1 Baseline characteristics of healthy subjects and patients with gastric esophageal ulcer, gastric ulcer, duodenal ulcer and gastric cancer *n* (%)

	HS (*n* = 165)	EU (*n* = 21)	GU (*n* = 136)	DU (*n* = 100)	GC (*n* = 89)
Age (yr)	51 ± 14	54 ± 12	63 ± 15[c]	54 ± 15	67 ± 14[c]
Sex (M/F)	83/82	17/4[b]	75/61	67/33[b]	63/26[b]
Smoking	20 (12)	6 (29)[a]	39 (29)[c]	37 (37)[c]	12 (13)
Alcohol drinking	12 (8)	2 (10)	4 (3)	9 (9)	6 (7)
Helicobacter pylori infection	68 (41)	6 (28)	76 (56)[a]	75 (75)[c]	45 (51)[a]

[a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ *vs* healthy subjects. HS: Healthy subjects; EU: Esophageal ulcer; GU: Gastric ulcer; DU: Duodenal ulcer; GC: Gastric cancer.

paraffin, and sectioned. The sections were stained with a haematoxylin and eosin stain and a modified Giemsa stain as previously described[18]. Sections were examined blinded to the patient's clinical diagnosis. The scores of acute inflammation (neutrophil infiltrate), chronic inflammation (mononuclear cell infiltrate), glandular atrophy, intestinal metaplasia and *H. pylori* density were graded from 0 to 3 as described by the updated Sydney system[19].

Statistical analysis

Statistical evaluations were performed using the SPSS program (version 10.1, Chicago, Illinois, USA). The differences in gastric juice acidity between HSs and patients with EU, GU, DU or GC were assessed by Student's *t*-test. The chi-square test with or without Yate's correction for continuity and Fisher's exact test, when appropriate, were applied to analyze the categorized variables. Differences were considered to be significant at $P < 0.05$. A multivariate analysis with logistic regression method was carried out to assess the independent factors influencing gastric acidity of gastric juices. The studied variables included the following: age (< 60 years or ≥ 60 years), gender, family history of gastric cancer (presence or absence), history of smoking (< 1 pack/wk or ≥ 1 pack/wk), history of alcohol consumption (< 80 g/d or ≥ 80 g/d), history of tea consumption (< 1 cup/d or ≥ 1 cup/d), coffee consumption (< 1 cup/d or ≥ 1 cup/d), bile stain of gastric juice, *H. pylori* status (presence or absence) and parameters of histological gastritis.

RESULTS

Table 1 shows the demographic characteristics of HSs and patients with EU, GU, DU and GC. Patients with GU and GC were significantly older than HSs (63 ± 15, 67 ± 14 years *vs* 54 ± 12 years, both $P < 0.001$). Additionally, the EU, DU and GC patient groups had higher male-to-female ratios than the HS group (all $P < 0.05$). No significant differences in history of alcohol consumption were identified between groups. However, the rates of cigarette smoking in EU, GU and DU patients were significantly higher than that of HSs ($P < 0.05$, 0.05 and 0.01, respectively). Furthermore, the rates of *H. pylori* infection in GU, DU and GC patients were also significantly higher than that of HSs ($P < 0.05$, < 0.001 and < 0.05, respectively).

Independent factors determining the acidity of gastric juice

Univariate analysis of 15 clinical and histological factors demonstrated that the following nine factors were significantly associated with hypoacidity: old age ($P < 0.001$), family history of GC ($P < 0.05$), bile reflux ($P < 0.001$), *H. pylori* infection ($P < 0.05$), intestinal metaplasia of the antrum ($P < 0.01$), and acute inflammatory score, chronic inflammatory score, atrophy and intestinal metaplasia of the corpus (all *P* values < 0.001, Table 2). Smokers had a lower frequency of gastric hypoacidity than non-smokers, and alcohol drinkers also had less hypoacidity than drinkers. However, the differences concerning smoking and drinking did not reach statistical significances ($P = 0.258$ and 0.100, respectively). Multivariate analysis with a stepwise forward logistic regression method disclosed only bile reflux, high acute inflammatory score of the corpus, and atrophy of the corpus were independent risk factors for the development of gastric hypoacidity with odds ratios of 3.1 (95% CI: 1.3-7.3), 3.1 (95% CI: 1.2-7.9) and 3.5 (95% CI: 1.3-9.2, Table 3).

The subjects with *H. pylori* infection had higher frequencies of high acute inflammatory score (76% *vs* 37%, $P < 0.001$) and high chronic inflammatory score (96% *vs* 74%, $P < 0.001$) in the antrum than those without *H. pylori* infection. Additionally, they also had higher frequencies of high acute inflammatory score (50% *vs* 31%, $P = 0.018$), high chronic inflammatory score (80% *vs* 60%, $P = 0.006$) and gland atrophy (38% *vs* 21%, $P = 0.028$) in the corpus than uninfected subjects.

Acidity of gastric juices in HSs and upper gastrointestinal diseases

Table 4 showed the pH levels of gastric juices in benign and malignant gastrointestinal diseases. EU and DU patients had a higher gastric acidity than HSs (1.91 ± 0.28 and 2.09 ± 0.09 *vs* 2.90 ± 0.16, both $P < 0.05$). In contrast, GU and GC patients had a lower gastric acidity than HSs (3.42 ± 0.20 and 6.62 ± 0.22 *vs* 2.90 ± 0.16, both $P < 0.001$). Overall, hypoacidity existed in 22%, 5%, 29%, 5% and 88% of HSs, EU, GU, DU and GC patients, respectively.

DISCUSSION

This work demonstrated the differences in acidity of

Table 2 Univariate analysis for clinical and histological factors related to the hypoacidity of gastric juice

Principal parameters	*n*	Rate of hypoacidity (%)	*P* value
Clinical factors			
Age (yr)			< 0.001
< 60	282	24.8	
⩾ 60	229	39.7	
Sex			0.496
Female	206	29.6	
Male	305	32.5	
Family history of gastric cancer			0.048
-	497	31.0	
+	14	50.0	
Smoking			0.259
-	396	33.3	
+	115	25.2	
Alcohol consumption			0.100
-	478	32.6	
+	33	15.2	
Bile stain of gastric juice			< 0.001
-	301	20.9	
+	210	46.7	
Helicobacter pylori infection			0.013
-	241	25.7	
+	270	36.7	
Histological factors			
Antrum			
Acute inflammatory score			0.476
Low (grade 0, 1)	61	44.3	
High (grade 2, 3)	91	38.5	
Chronic inflammatory score			0.292
Low (grade 0, 1)	20	30.0	
High (grade 2, 3)	132	42.4	
Atrophy			0.195
-	43	32.6	
+	109	44.0	
Intestinal metaplasia			0.006
-	91	31.9	
+	61	54.1	
Corpus			
Acute inflammatory score			< 0.001
Low (grade 0, 1)	88	28.4	
High (grade 2, 3)	64	57.8	
Chronic inflammatory score			< 0.001
Low (grade 0, 1)	43	18.6	
High (grade 2, 3)	109	49.5	
Atrophy			< 0.001
-	106	28.3	
+	46	69.6	
Intestinal metaplasia			< 0.001
-	126	34.9	
+	26	69.2	

Table 3 Multivariate analysis for independent factors determining hypoacidity of gastric juice

Risk factors	Coefficient	SE	OR (95% CI)	*P* value
Bile reflux	1.116	0.446	3.1 (1.3-7.3)	0.012
Acute inflammatory score of the corpus	1.115	0.488	3.1 (1.2-7.9)	0.022
Atrophy of the corpus	1.245	0.497	3.5 (1.3-9.2)	0.012

gastric juices among patients with upper gastrointestinal diseases. EU and DU patients had a higher gastric acidity than HSs. In contrast, GU and GC patients had a lower gastric acidity than HSs. This study is the first to verify higher gastric acidity in EU patients compared with HSs. In this study, only 5% of EU patients possessed hypoacidity of gastric juice, whereas gastric hypoacidity existed in 22%, 29% and 88% of HSs, GU and GC patients, respectively. The data imply that normal or higher acidity of gastric juice is an important factor for the development of GERD besides lower esophageal pressure abnormalities, hiatal hernia and delayed gastric emptying[20].

Several histological studies also showed chronic atrophic gastritis present in 80%-90% of GC patients[21]. In this study, gastric hypoacidity existed in 88% of the patients with GC. This finding indicates atrophic gastritis with gastric hypoacidity is a crucial step for the development of gastric adenocarcinoma. *H. pylori* infection, old age and cagA and vacA m1 positivity have been identified as independent risk factors for the development of atrophic gastritis[22]. We propose that the high prevalence of *H. pylori* infection, advanced age, some bacterial virulent factors and susceptible host factors may contribute to the development of gastric atrophy and hypoacidity of the GC patients in this study.

The current work also showed that DU patients had a higher gastric acidity than HSs. This result supported previous observations demonstrating increased basal and stimulated acid secretion by the body of the stomach and increased acid load in the duodenum in patients with DU[23]. On the contrary, GU patients in this study had a lower gastric acidity than HSs, suggesting that mucosal defensive impairments are more important than increased acid load in the pathogenesis of GU. The findings were consistent with previous reports[24] revealing that the majority of gastric ulcers do not have increased gastric acid secretion.

Multivariate analysis in this study revealed that bile stain of gastric juice, high acute inflammatory score and atrophy of the corpus were independent factors for the development of gastric hypoacidity. The atrophy of the corpus was the most important factor for gastric hypoacidity with an odds ratio of 3.5. Since gastric acid is secreted by the parietal cells in the corpus, gland atrophy of the corpus leading to hyposecretion of acid and gastric hypoacidity is logical.

In 1988, Correa *et al*[25] proposed a human model of gastric carcinogenesis that gastric cancers develop through a complex sequence of events from normal mucosa to superficial gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and finally to intestinal-type adenocarcinoma[21,26,27]. Gland atrophy resulting in hypochlorhydria is a key step in this theory and accounts for gastric bacterial colonization, reduction of dietary nitrates to nitrites and the formation of potentially carcinogenic N-nitroso compounds[4,25]. In this study, *H. pylori*-infected patients had higher frequencies of gland atrophy in the corpus (38% *vs* 21%) and gastric hypoacidity (37% *vs* 26%) than uninfected subjects. Additionally, they also had stronger acute and chronic inflammation in the corpus than uninfected

Table 4 pH levels of gastric juices in upper gastrointestinal diseases *n* (%)

pH of gastric juice	HS (*n* = 165)	EU (*n* = 21)	GU (*n* = 136)	DU (*n* = 100)	GC (*n* = 89)
Level					
pH < 2	82 (50)	19 (91)	33 (24)	55 (55)	5 (5)
2 ≤ pH < 3.5	46 (28)	1 (5)	63 (46)	40 (40)	6 (7)
3.5 ≤ pH < 4.0	6 (4)	0 (0)	5 (4)	2 (2)	5 (6)
4.0 ≤ pH < 5.0	5 (3)	0 (0)	7 (5)	0 (0)	5 (6)
5.0 ≤ pH < 6.0	5 (3)	0 (0)	3 (2)	1 (1)	2 (2)
6.0 ≤ pH < 7.0	2 (1)	0 (0)	2 (2)	1 (1)	9 (10)
7 ≤ pH	19 (12)	1 (5)	23 (17)	1 (1)	57 (65)
mean ± SE	2.90 ± 0.16	1.91 ± 0.28[a]	3.42 ± 0.20[b]	2.09 ± 0.09[a]	6.62 ± 0.22[b]

[a]$P < 0.05$, [b]$P < 0.001$ *vs* healthy subjects. HS: Healthy subjects; EU: Esophageal ulcer; GU: Gastric ulcer; DU: Duodenal ulcer; GC: Gastric cancer.

subjects. These findings suggest that *H. pylori* infection is an important factor contributing to the development of atrophic gastritis in the corpus and hypo-secretory status of the stomach.

Primary duodenogastric reflux may occur due to antroduodenal motility disorder or incompetent pyloric sphincter[28]. The retrograded bile and duodenal contents can induce damage of the gastric mucosa[29]. It has been observed that duodenogastric reflux plays a crucial role in the pathogenesis of alkaline gastritis and GU[29]. Since the presence of bile in the gastric juice implies retrograde passage of alkaline duodenal contents into the stomach, it is reasonable to expect increased pH levels of gastric juice in the subjects with bile in gastric juice.

A higher degree of acute inflammation was the other histological factor predicting gastric hypoacidity in this study. Currently, we have no definite rationale to explain the association between dense neutrophil infiltrate and increased pH level in gastric juices, but dense neutrophil infiltrates may reflect the high density of *H. pylori* in the stomach[30], and have also been reported as one of the important factors related to the progression of atrophic gastritis[26].

In this study, smokers had a trend of less hypoacidity than non-smokers. The finding was supported by previous studies showing that nicotine increases acid secretion and decreases prostaglandin synthesis[31]. It is interesting to note that alcohol drinkers also had a trend of less hypoacidity than nondrinkers. The reasons for this finding remain unclear, but some studies demonstrated that fermented and nondistilled alcoholic beverages increase gastrin levels and acid secretion[32]. Additionally, succinic and maleic acid contained in certain alcoholic drinks also stimulate acid secretion[32].

In conclusion, bile reflux, atrophy and neutrophil infiltration of the corpus are three independent factors determining the acidity of gastric juices. Gastric acidities in patients with various upper gastrointestinal diseases are quite different. EU and DU patients have a higher gastric acidity whereas GU and GC patients have a lower gastric acidity compared with HSs.

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COMMENTS

Background

The acidity of gastric juice is one of the crucial factors in the development of most upper gastrointestinal diseases.

Research frontiers

The authors designed this study to search the independent factors determining gastric juice acidity and to investigate the acidity of gastric juices in various upper gastrointestinal diseases.

Innovations and breakthroughs

This study is the first to verify higher gastric acidity in esophageal ulcer patients compared with healthy subjects. The results showed that bile stain of gastric juice, high acute inflammatory score and atrophy of the corpus were independent factors for the development of gastric hypoacidity.

Applications

Results of this study are helpful for understanding the pathogenesis of upper gastrointestinal diseases and the factors influencing the acidity of gastric juice.

Terminology

The updated Sydney System is a schema for the classification and grading of histological gastritis established by an International Workshop on the Histopathology of Gastritis, Houston 1994.

Peer review

The study shows evidence collected in large population, however, there is lack of the definite conclusion in this paper. Instead of that, the authors made a comment in conclusion on the results, not providing the idea about the potential mechanism of changes in the acidity of gastric secretion in different group of patients. The Discussion is too general and not always linked to results obtained.

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BRIEF ARTICLE

FOXP3 expression and clinical characteristics of hepatocellular carcinoma

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Abstract

AIM: To study the biological and clinical characteristics of transcription factor forkhead box protein 3 (FOXP3) in hepatocellular carcinoma (HCC).

METHODS: We analyzed the expression and localization of FOXP3 in HCC tissues and cell lines to evaluate its biological features. The relationship between FOXP3 staining and clinical risk factors of HCC was assessed to identify the clinical characteristics of FOXP3 in HCC.

RESULTS: The mRNA and protein expression of FOXP3 were found in some hepatoma cell lines. Immunohistochemical (IHC) analysis of HCC sections revealed that 48% of HCC displayed FOXP3 staining, but we did not find any FOXP3 staining in normal liver tissues and para-tumor tissues. IHC and Confocal analysis showed that the expressions of FOXP3 were mainly present in the nucleus and cytoplasm of tumor cells in tissues or cell lines. In HCC, the distribution of FOXP3 was similar to that of the cirrhosis, but not to the hepatitis B virus. Those findings implicate that FOXP3 staining seems to be associated with the high risk of HCC.

CONCLUSION: The clinical characteristics of FOXP3 in HCC warrants further studies to explore its functions and roles in the cirrhosis and development of HCC.

Key words: Forkhead box protein 3; Hepatocellular carcinoma; Tumor differentiation; Cirrhosis; Hepatitis B virus

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INTRODUCTION

Hepatocellular carcinoma (HCC), which consists predom-

inantly of primary liver cancer, is the fifth most common malignancy in men and the eighth one in women worldwide. The number of new cases of HCC is about 564 000 per year[1]. Cirrhosis and virus infection, such as hepatitis B virus (HBV) and hepatitis C virus (HCV), are the major known risk factors for HCC[2,3]. HCC has a poor prognosis and a low survival rate in the majority of patients[4]. The current treatment options of HCC include surgical resection, liver transplantation and local ablative therapy, which are effective only in limited tumors[5]. To improve the treatment of HCC will require a better understanding of the biological development and molecular events in the immune system of HCC.

Forkhead box protein 3 (FOXP3) is a member of the forkhead/winged-helix family of transcriptional regulators and is highly conserved in normal cells. The full-length protein contains 431 amino acids. *Foxp3* is considered to be an important gene of thymically derived and naturally occurring regulatory T cells (Tregs)[6]. Mutations in human *Foxp3* are associated with immune diseases, such as multi-organ autoimmune disorder, immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome (IPEX)[7], in which Tregs from affected patients are greatly reduced in number and suppressive activity[8-10]. A high prevalence of Tregs is thought to be an unfavorable prognostic indicator for HCC[11].

Recent publications described the expression of FOXP3 in pancreatic carcinoma cells, melanoma cells and other tumor cells[12-14]. It has been found that FOXP3 expression was related to the regulation of several cytokines, such as IL-10 and TGF-β2, and FOXP3 might mediate the inhibiting efficacy of tumor cells to escape immune destruction. Those reports implicated that FOXP3 performs its functions in the regulation of tumor progression by expressing not only in Tregs, but also in tumor cells. We assumed that FOXP3 may also be functional in tumor cells of HCC.

This study was designed to investigate whether expression of FOXP3 transcripts and mature protein is related to HCC. We also evaluated the distribution of FOXP3 in human HCC tissues and its relationship with the diagnosis, differentiation and clinical risk factors of HCC.

MATERIALS AND METHODS

Source of normal and cancerous liver tissue sections and tissue array

Normal (8) and cancerous liver tissues (21) were obtained from HCC patients who underwent resection of liver. The circulating HBV markers and ultrasound examination were performed regularly. The quantitative cirrhosis score was derived from the ultrasonographic evaluation of the liver surface, liver parenchyma, caliber of intrahepatic blood vessels and spleen size. Cirrhosis scores of $\geqslant 7$ were used to define cirrhosis[15]. Normal controls were histologically normal tissues obtained from patients who underwent partial hepatectomy for metastatic tumor or liver biopsy. Microarray tissues were obtained from Cybrdi, USA. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki in a prior approval by the Fourth Military Medical University, China.

Cell culture

Complete medium (RPMI-1640) contained RPMI-1640 supplemented with 2 mmol/L Glutamax, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mmol/L HEPES (Invitrogen, USA) and 10% FCS (Thermo Trace, Australia). The following cell lines were obtained from the Biotechnology Center of the Fourth Military Medical University, such as SMMC-7721 and Hepa-G2. All tumor cell lines were maintained in complete RPMI-1640 and passaged using trypsin/EDTA (Invitrogen, USA). *Foxp3* transiently transferred 293 cells were established. Melanocytes were freshly prepared when used (derived from normal human skins from the Department of Dermatology of Xijing Hospital of the Fourth Military Medical University).

Semi-quantitative and reverse-transcription polymerase chain reaction

Total RNA was isolated from HCC cells and melanocyte (as control) using Trizol reagent (Invitrogen, USA). A total of 500 ng RNA was reversely transcribed using the Kit from Takara, Japan. The polymerase chain reaction (PCR) was performed for *Foxp3* fragment amplification. The following primers were used (5'-3'): *Foxp3* sense: CACAACATGCGACCCCCTTTCACC; *Foxp3* antisense: AGGTTGTGGCGGATGGCGTTCTTC. β-actin was used as an internal control for normalization (primer sequences available on request). Semi-quantitative PCR of *Foxp3* transcripts was done by comparing the signal intensity of PCR product of *Foxp3* gene with that of β-actin gene from the same RNA sample using agarose gel electrophoresis. The intensity of the product band was quantified by densitometric scanning of the gel (Pharmacia Biotech) using "Total image" 1D GEL ANALYSIS software. DNA marker (Takara, Japan) was run in each gel to confirm the size of PCR product.

Western blotting analysis

To examine the protein expression level of FOXP3 in HCC, whole cell lysate was subjected to SDS-PAGE electrophoresis followed by blotting on a nitrocellulose (NC) membrane. During FOXP3 detection, membranes were probed with goat anti-human FOXP3 polyclonal antibody at 4℃ overnight followed by incubation with a secondary horseradish peroxidase-conjugated antibody. The mouse anti-human β-actin monoclonal antibody was used as an internal control (R&D, USA). Chemiluminescent detection was done with the enhanced chemiluminescence detection kit (Anmei, China).

Flow cytometric analysis

To determine the expression levels of FOXP3, hepatoma cell lines were stained for FOXP3 and analyzed by flow cytometry. Cells were washed in PBS containing 1% bovine serum albumin (BSA) and 0.1% NaN_3 before antibody staining followed by fixation with 1% paraformaldehyde.

Fluorescein isothiocyanate (FITC)-conjugated rat anti-human FOXP3 monoclonal antibody was purchased from eBiosciences, USA. A total of 10^5 events were collected using Becton Dickinson FACScaliber (Becton Dickinson, USA). Analysis was performed using the WinMDI 2.8 program (Purdue University Cytometry Laboratories, USA).

Confocal microscopic analysis

For double-label immunofluorescence, formalin-fixed cell line slides were treated in 3% hydrogen peroxide in methanol for 10 min. Following three rinses in PBS, the slides were treated in blocking solution (Zhongshan, China) for 1 h, and then incubated with fluorescein isothiocyanate (FITC)-conjugated rat anti-human FOXP3 monoclonal antibody (eBioscience, USA) for another 1 h at room temperature. After rinsed in PBS, the slides were treated with a 1:1000 dilution of diamidino-phenyl-indole (DAPI) (stock solution: 1 mg/mL) (Sigma-Aldrich, USA) for 30 min. DAPI staining was used to visualize nuclei. The slides were mounted with the anti-fade mounting medium. Slides were examined under a Leica TCS-SP laser scanning confocal microscope (Leica, German). All images were collected using a pinhole of 1 Airy.

Immunohistochemistry

Paraffin-embedded resected liver cancer specimens were provided by Xijing Hospital tissue bank. Rat anti-human FOXP3 monoclonal antibody was applied to paraffin-embedded sections after microwave antigen retrieval for 10 min in citrate buffer (pH 6.0). Specimens were treated with 0.3% hydrogen peroxide in methanol for 15 min after incubation with the primary antibody to block endogenous peroxidase activity. The secondary antibody of horseradish peroxidase-labeled goat anti-rat antibody (Zhongshan, China) was incubated for 1 h. These slides were examined systematically using an image analyzer system (Olympus BH-2 microscope, Japan).

Semi-quantitative analysis of IHC

Slides were reviewed under light microscopy by two pathologists separately. Semi-quantitative analysis of FOXP3 staining was assessed as 0, 1+, 2+, and 3+ as previously established[16,17]. Grade 0 was defined as the complete absence or weak FOXP3 staining in < 1% of the tumor cells; grade 1+ was focal FOXP3 staining in 1%-10% of tumor cells; grade 2+ was positive FOXP3 staining in 11%-50% of tumor cells; and grade 3+ was positive FOXP3 staining in > 50% of tumor cells. A global assessment of the entire tumor was made without selection for the invasive front or areas of active tumor growth. The frequency and semi-quantitative analysis of positive tumors for all regions were calculated for statistical comparisons.

Statistical analysis

Differences in proportions were compared by a Pearson Chi-square test or Fisher's exact test as appropriate. The statistical correlation between the grades of HCC differentiation and the staining level of FOXP3 was analyzed by the Cochran-Mantel-Haenszel test. The dependability between the distribution of cirrhosis or HBV infection and FOXP3 expression was evaluated by *t* test. Differences with a *P* value less than 0.05 were considered to be statistically significant. All analyses were done using SAS statistical software version 9.1 (Cary, USA).

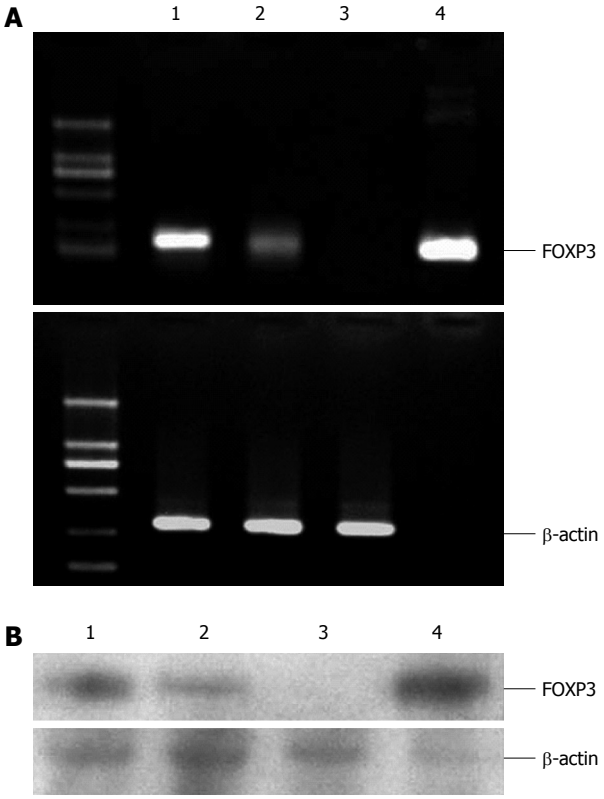

Figure 1 Expression of forkhead box protein 3 in hepatoma cell lines. A: Expression of forkhead box protein 3 (FOXP3) mRNA is different in hepatoma cell lines by reverse-transcription polymerase chain reaction. β-actin was used to verify the integrity of the template cDNA preparations. 1: SMMC-7721, 2: Hepa-G2, 3: Melanocytes, 4: Foxp3-plasmid; B: Expression of FOXP3 protein was different in hepatoma cell lines by Western blotting. Melanocytes served as a negative control. Foxp3 transiently transferred 293 cells were established as a positive control. 1: SMMC-7721, 2: Hepa-G2, 3: Melanocytes, 4: Foxp3/293 cells.

RESULTS

FOXP3 expression in hepatoma cell lines

In RT-PCR analysis, *Foxp3* was found in some hepatoma cell lines. To further validate the changes of *Foxp3*, semi-quantitative RT-PCR analysis from two hepatoma cell lines and melanocytes were conducted. The results revealed a significant overexpression of *Foxp3* in HCC specimens as compared with nonmalignant melanocytes (Figure 1A). The cell lines shown in Figure 1A (SMMC-7721 and Hepa-G2) were uniformly positive for *Foxp3* transcription. But we did not find any *Foxp3* transcription in melanocytes as expected. *Foxp3* full length plasmid was constructed and performed as a positive control.

Furthermore, as shown in Figure 1B, FOXP3 protein expression could be detected in hepatoma cell lines by Western blotting. Because it was the first time to dem-

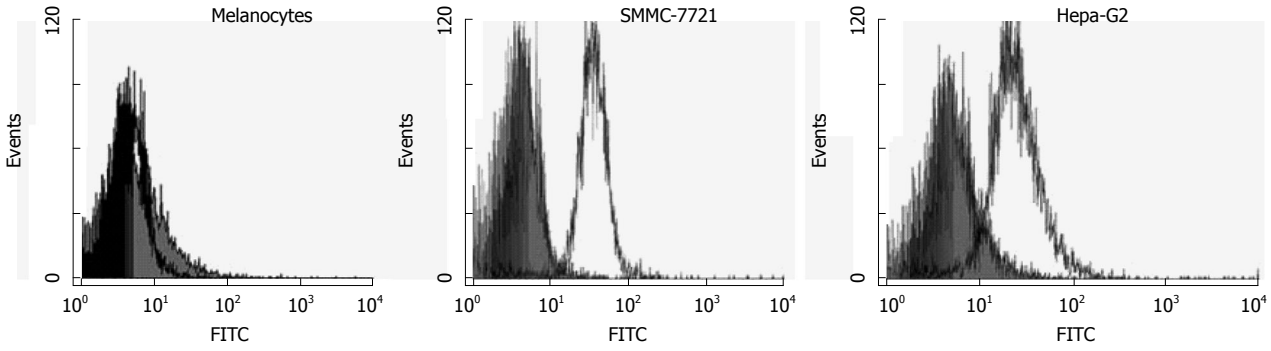

Figure 2 Flow cytometry of forkhead box protein 3 expression in various hepatoma cell lines. Melanocytes served as a negative control. The grey underlaid plot represents staining with the isotype, and the white underlaid plot represents staining with anti-human forkhead box protein 3 antibody. FITC: Fluorescein isothiocyanate.

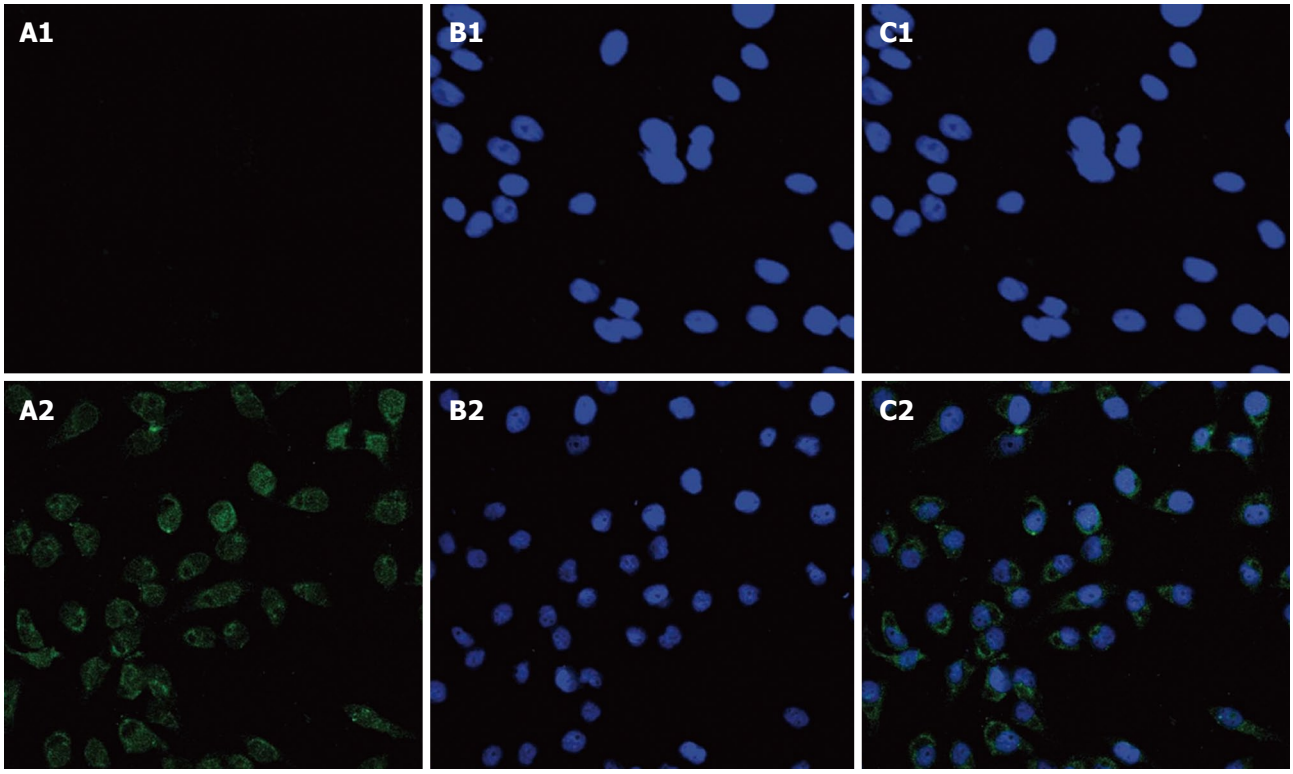

Figure 3 Double-label immunofluorescence analysis of forkhead box protein 3 expression in hepatoma cell line. A: SMMC-7721 cells express forkhead box protein 3 (FOXP3) in both nuclei and perinuclear cytoplasm (imaged with green fluorescent) (A1: Melanocytes FOXP3-FITC, A2: SMMC-7721 FOXP3-FITC); B: Imaged with diamidino-phenyl-indole (DAPI) to identify the nuclei of SMMC-7721 and melanocytes (B1: Melanocytes DAPI, B2: SMMC-7721 DAPI); C: Image superimposed on a differential interference contrast background confirmed colocalization (C1: Melanocytes merge, C2: SMMC-7721 merge).

onstrate FOXP3 expression in liver cells, we confirmed the staining results in Western blotting with two different anti-FOXP3 antibodies (eBioscience and R&D, USA). We found that the intensity of FOXP3 expression varied in different cell lines. In this experiment, *Foxp3* transiently transferred 293 cells were established as a positive control cell line.

Localization of FOXP3 in hepatoma cell lines

FOXP3 FITC-staining resulted in a shift in the fluorescence of the entire population of hepatoma cell lines compared with the isotype control in the results of flow cytometry. In contrast, melanocytes did not express FOXP3, as shown in Figure 2. Confocal microscopy was used to examine the distribution of FOXP3 in hepatoma cell lines and melanocytes. In these experiments, the nuclei were stained with DAPI to facilitate analysis. The results showed that SMMC-7721 cells exhibited intense nucleic and less cytoplasmic FOXP3 expression (Figure 3).

Expression and distribution of FOXP3 in HCC tissues

IHC was used to analyze the protein expression and localization of FOXP3 in HCC tissues. FOXP3 staining was done on a set of 29 tissue sections (Table 1) and a tissue array containing 154 cores (Table 2). Those samples were selected from normal and cancerous HCC tissues.

Table 1 Association of forkhead box protein 3 expression with pathologic grades in 21 hepatocellular carcinoma tissue sections by immunohistochemical *n* (%)

Demographic or clinical characteristic	No. of tumor specimens (*n* = 21)	FOXP3		*P*	FOXP3 immunohistochemistry intensity score				*P*
		Positive	Negative		0	1	2	3	
Gender									
Male	20	10 (50)	10 (50)	1.0000[1]	10 (50)	7 (35)	3 (15)	0 (0)	1.0000[1]
Female	1	0 (0)	1 (100)		1 (100)	0 (0)	0 (0)	0 (0)	
Age (yr)									
> 60	4	3 (75)	1 (25)	0.3108[1]	1 (25)	1 (25)	2 (50)	0 (0)	0.1031[1]
≤ 60	17	7 (41)	10 (59)		10 (59)	6 (35)	1 (6)	0 (0)	
Differentiation grade									
Well	7	4 (57)	3 (43)	1.0000[1]	3 (43)	3 (43)	1 (14)	0 (0)	1.0000[1]
Moderate	7	3 (43)	4 (57)		4 (57)	2 (29)	1 (14)	0 (0)	
Poor	7	3 (43)	4 (57)		4 (57)	2 (29)	1 (14)	0 (0)	
Tumor	21	10 (48)	11 (52)	0.0265[1,a]	11 (52)	7 (33)	3 (15)	0 (0)	
Normal	8	0 (0)	8 (100)		8 (100)	0 (0)	0 (0)	0 (0)	

[1]Fisher's exact test. [a]$P < 0.05$. FOXP3: Forkhead box protein 3.

Table 2 Association of forkhead box protein 3 expression with pathologic grades in 140 hepatocellular carcinoma tissue arrays by immunohistochemical *n* (%)

Demographic or clinical characteristic	No. of tumor specimens (*n* = 140)	FOXP3		*P*	FOXP3 immunohistochemistry intensity score				*P*
		Positive	Negative		0	1	2	3	
Gender									
Male	119	28 (24)	91 (76)	0.6193[2]	91 (76)	20 (17)	7 (6)	1 (1)	0.4895[1]
Female	21	6 (28)	15 (72)		15 (72)	3 (14)	3 (14)	0 (0)	
Age (yr)									
> 60	30	9 (30)	21 (70)	0.4103[2]	21 (70)	4 (13)	5 (17)	0 (0)	0.1542[1]
≤ 60	110	25 (23)	85 (77)		85 (77)	19 (17)	5 (5)	1 (1)	
Differentiation grade									
Well	32	11 (34)	21 (66)	0.2410[2]	21 (66)	8 (25)	2 (6)	1 (3)	0.2887[1]
Moderate	73	14 (19)	59 (81)		59 (81)	8 (11)	6 (8)	0 (0)	
Poor	35	9 (26)	26 (74)		26 (74)	7 (20)	2 (6)	0 (0)	
Tumor	140	34 (24)	106 (76)	0.0405[1,a]	106 (76)	23 (16)	10 (7)	1 (1)	
Normal	14	0 (0)	14 (100)		14 (100)	0 (0)	0 (0)	0 (0)	

[1]Fisher's exact test; [2]Pearson χ^2. [a]$P < 0.05$. FOXP3: Forkhead box protein 3.

Pathologists evaluated the expression level of FOXP3 in different HCC tissue samples according to the percentage of FOXP3 staining (Figure 4). Ten of 21 (48%) HCC tissue sections displayed FOXP3 staining. Interestingly, we noticed that FOXP3 was mainly localized in the nucleus of well differentiated HCC tissues and cytoplasm of moderately and poorly differentiated HCC tissues in tissue sections and array. To confirm the validity of the observation, we used two different anti-human FOXP3 antibodies (eBioscience and R&D, USA) to repeat the experiments. Both antibodies gave similar patterns about FOXP3 localization in HCC tissues.

Occurrence of FOXP3 is not associated with differentiation

The expression of FOXP3 at each histopathological grade were examined and recorded in all the patients. It is interesting that many cancerous patients were found to be FOXP3 positive, whereas the intensity of FOXP3 staining was low. The proportion of FOXP3 staining cells had no differences among the histopathological grades in HCC tissue array. In this experiment, 11 of 32 grade Ⅰ (34%), 14 of 73 grade Ⅱ (19%), and 9 of 35 grade Ⅲ (26%) carcinoma cores (Figure 5) were positive in FOXP3 staining ($P = 0.2410$, Pearson χ^2 test; Table 2). This result implicated that FOXP3 staining might be a useful prognostic factor, but not a clue in differentiation of HCC patients. We also found that normal liver tissues were devoid of FOXP3 expression (Figures 4 and 5). In addition, there was no relationship between FOXP3 expression and gender or age, no matter in tissue section or array (Tables 1 and 2).

Relationship between HCC risk factors and FOXP3 expression in tumor tissues

HCC is a heterogeneous tumor. Cirrhosis, HBV and HCV infections represent the major known risk factors for HCC. Those factors could cooperate or act alone to promote the incidence of HCC depending on the different pathways and molecules.

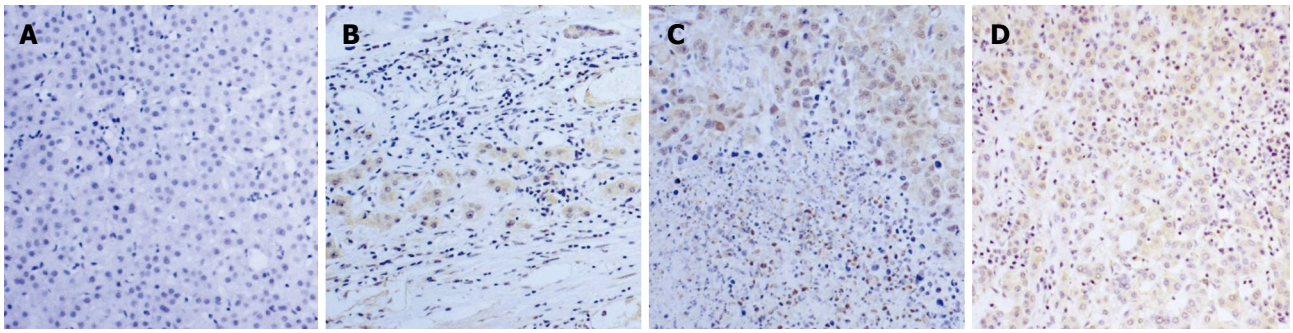

Figure 4 Hepatocellular carcinoma immunohistochemical score (× 100). Forkhead box protein 3 (FOXP3) expression in hepatocellular carcinoma (HCC) by immunohistochemical (IHC). Images represent HCC tissues with IHC scores of 0 (A), 1 (B), 2 (C) and 3 (D). In IHC score 0, many FOXP3-positive lymphocytes are tumor-infiltrating Tregs.

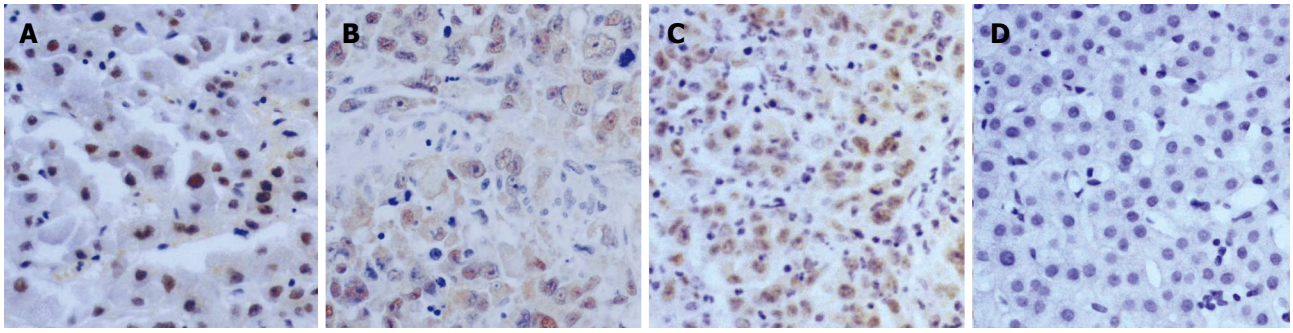

Figure 5 Hepatocellular carcinoma immunohistochemical staining (× 200). Immunohistochemical (IHC) staining of paraffin embedded hepatocellular carcinoma (HCC) tissues revealed different levels of forkhead box protein 3 (FOXP3) expression in HCC cells (PCH101 antibody, eBioscience). A: Well-differentiated HCC (nuclear FOXP3 staining); B: Moderately-differentiated HCC (cytoplasmic FOXP3 staining); C: Poorly-differentiated HCC (cytoplasmic FOXP3 staining); D: Normal liver tissue (nuclear FOXP3 staining in Tregs).

Table 3 Relationship between hepatitis B virus infection (or cirrhosis) distribution and forkhead box protein 3 expression in 21 hepatocellular carcinoma samples

		FOXP3 expression		*P*
		-	+	
HBV infection	-	2	1	0.021[a]
	+	9	9	
Cirrhosis	-	1	3	0.092
	+	10	7	

FOXP3: Forkhead box protein 3; HBV: Hepatitis B virus. [a]$P < 0.05$.

We detected the circulating HBV markers and liver cirrhosis levels to analyze the relationship between the expression of FOXP3 and HBV/cirrhosis to evaluate the potential clinical characteristics of FOXP3.

As shown in Table 3, among the HCC patients, 42.9% HBV infection and 33.3% cirrhosis of the section samples were FOXP3 positive. Statistical results revealed that FOXP3 expression coincided with the occurrence of cirrhosis ($P = 0.092$), while it is highly significant with HBV infection ($P = 0.021$) in HCC patients.

DISCUSSION

Although the factors and molecular events associated with the progression of HCC are complex and not well established, FOXP3 has been shown to play an important role in Tregs in HCC invasion[1,5,11]. In this study, we assessed the expression and subcellular localization of FOXP3 in hepatoma cell lines and HCC tissues to identify the fact that FOXP3 is expressed by tumor cells. We also identified some clinical characteristics of FOXP3 in HCC.

Few studies assessing FOXP3 expression in tumor tissues and cell lines have been reported. Hinz *et al*[12] described for the first time the expression and function of FOXP3 in pancreatic ductal adenocarcinoma cells and tissues. They detected FOXP3 expression in tumor cells of 24/39 patients with pancreatic carcinoma. Although they were unable to find a correlation between FOXP3 expression and tumor stage or survival rate, their findings indicate that pancreatic carcinoma cells share growth suppressive effects with Tregs depending on the function of FOXP3. Their results also suggest that FOXP3 may promote tumor cells to mimic characters of Tregs to represent an immune evasion function in microenvironment. Ebert *et al*[13] and Karanikas *et al*[14] reported that FOXP3 transcription factor was expressed by melanoma cells and lots of tumor cells. These evidences suggest that FOXP3 is related to tumor escape and can be used as a potential tumor antigen.

In agreement with their findings, *Foxp3* transcription and protein expression were found in hepatoma cell lines

(SMMC-7721 and Hepa-G2) in this study. After Confocal analysis, we noticed that nuclear and less cytoplasm FOXP3 expression was more prevalent in hepatoma cell lines. To our knowledge, this study provides the first evidence of nuclear and cytoplasm localization of FOXP3 in hepatoma cell lines. In addition, we found that FOXP3 was mainly expressed in the nucleus in well differentiated HCC tissues and cytoplasm in moderately and poorly differentiated HCC tissues. It implicates that the factors (such as cytokines, immune cells, ligand and receptor of tumor cells) coming from microenvironment and differentiation of tumor might induce a change in the subcellular localization of FOXP3 between cytoplasmic and nuclear expression patterns, which would result from post-translational modifications[18]. Therefore, the heterogeneous subcellular localization of FOXP3 in hepatoma cell lines and HCC tissues may reflect the different post-translationally modified forms of FOXP3. In particular, previous reports revealed the interaction of FOXP3 with nuclear factor of activated T cells (NFAT, a transcription factor) in Tregs[19]. It revealed that FOXP3 plays a key role in the formation of nuclear complexes that are important to regulate the transcription of functional genes[20].

In this study, we also found some clinical characteristics of FOXP3 in HCC. The statistical results revealed that HBV infection had different distribution patterns compared with the expression of FOXP3 in HCC. In our previous study, FOXP3 was not found in the hepatitis and normal liver cells. Therefore, we may draw a conclusion that FOXP3 of tumor cells has little relationship with the inflammation induced by HBV infection alone. But because of the limited number of patients, this still needs further studies to validate. On the other hand, 70% of FOXP3 positive HCC cases were companied with liver cirrhosis. This indicates that the pathogenesis and molecules of cirrhosis may have some effects on the regulation of FOXP3 expression. FOXP3 may be involved in the progression of cirrhosis-induced HCC.

On the contrary, Zou *et al*[21,22] reported that functional somatic mutations and down-regulation of the *Foxp3* gene were commonly found in human breast cancer tissues. The expression of FOXP3 was correlated with HER-2/ErbB2 and SKP-2 overexpression. So the function and mechanism of FOXP3 may be different in various tumor cells, which still remains to be elucidated.

In conclusion, this is the first report of FOXP3 staining in hepatoma cell lines and HCC tissues, creating a new focus that FOXP3 is widely expressed in tumor cells and tissues. As the molecular mechanisms of FOXP3 still remain unclear, the real function of FOXP3 in different tumors need to be further studied so as to understand the critical molecular events associated with tumor progression.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors. It is highly resistant to many conventional treatments and has a poor prognosis. forkhead box protein 3 (FOXP3) is considered to play an important role in naturally occurring regulatory T cells (Tregs). Recent reports have implicated that FOXP3 might have different effects on tumor cells. But the expression and mechanism of FOXP3 in HCC cells remain unclear.

Research frontiers

Few reports showed the expression of FOXP3 in pancreatic carcinoma cells, melanoma cells and other tumor cells. The reports found that FOXP3 expression was related to the regulation of several cytokines, such as IL-10 and TGF-β2. FOXP3 might mediate the immune inhibiting efficacy of tumor cells to escape immune destruction. The authors assumed that FOXP3 may also be functional in HCC.

Innovations and breakthroughs

Interestingly, the authors found that hepatoma cell lines expressed FOXP3 mainly in nuclear and cytoplasm. But FOXP3 was almost expressed in nuclear in well differentiated HCC tissues and cytoplasm in moderately and poorly differentiated HCC tissues. It suggests that the microenvironment and differentiation of tumor might induce a change in the subcellular localization of FOXP3 between cytoplasmic and nuclear expression patterns. Therefore, the heterogeneous subcellular localization of FOXP3 in hepatoma cell lines and tissues may reflect the different post-translationally modified forms of FOXP3. The authors also found that the distribution of FOXP3 was similar to that of the cirrhosis, but not to that of HBV infection in HCC. Although the function of FOXP3 in HCC is not clear, the result suggests that FOXP3 may be involved in the cirrhosis-induced HCC. Altogether, those findings implicate that FOXP3 staining seems to be associated with a high risk in HCC.

Applications

This study is creating a new focus that FOXP3 is widely expressed in HCC cells and tissues. However, the real function of FOXP3 in HCC needs to be further studied so as to understand the critical molecular events in HCC progression.

Peer review

The manuscript by Wang *et al* provides evidences supporting FOXP3 expression both in human hepatoma cells and in HCC tumors. Since this is the first time that expression of FOXP3 has been reported in liver cancer, the information is of interest.

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BRIEF ARTICLE

Cyclooxygenase-2 polymorphisms and susceptibility to gastric carcinoma: A meta-analysis

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Abstract

AIM: To investigate the association of the cyclooxygenases-2 (COX-2) polymorphisms and susceptibility to gastric cancer (GC) by means of meta-analysis.

METHODS: Publications addressing the association between polymorphisms of COX-2 and susceptibility to GC were selected from the MEDLINE, EMBASE and CBMdisc databases. Data was extracted from the studies by 2 independent reviewers. The meta-analyses were performed by RevMan 5.0.23. From these data, odds ratio (OR) with 95% confidence interval (CI) was calculated.

RESULTS: Ten studies were retrieved reporting a total of 11 COX-2 polymorphisms. Carriers of -765C, -1195A, -1290G, *2430T alleles and *429TT genotype revealed increased risk for GC (OR = 1.71, 95% CI: 1.01-2.90, *P* = 0.05; OR = 1.58, 95% CI: 1.05-2.38, *P* = 0.03; OR = 1.55, 95% CI: 1.01-2.39, *P* = 0.05; OR = 2.62, 95% CI: 1.20-5.73, *P* = 0.02 and OR = 0.74, 95% CI: 0.59-0.95, *P* = 0.02, respectively).

CONCLUSION: The -765C, -1195A, -1290G, *2430T alleles and *429TT genotype of COX-2 polymorphisms were determined a significant association with susceptibility to GC.

Key words: Cyclooxygenases-2; Gastric cancer; Polymorphisms; Susceptibility; Meta-analysis

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INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related death worldwide[1-3]. In 2008, around 21 500 people were diagnosed with gastric cancer and approximately 135 130 died of the disease in the United States[4]. GC is a complex and multifactorial disease. The marked geographic variation, time trends, and the migratory effect on GC incidence suggest that environmental or lifestyle factors are major contribu-

tors to the etiology of this disease. Examples include: diet, lifestyle, *Helicobacter pylori* (*H. pylori*) infection and genetic material[1].

Recent data has expanded upon the concept that inflammation is a critical component of tumor progression. Many cancers arise from sites of infection, chronic irritation and inflammation[5]. Tumor microenvironment is largely orchestrated by inflammatory cells, which are an indispensable participant in the neoplastic process, fostering proliferation, survival and migration. Inflammation also plays an important role in the development and progression of GC[6]. Epidemiological and animal data revealed that the use of non-steroidal anti-inflammatory drugs (NSAIDs) might reduce the risk of GC[7]. NSAIDs primarily inhibit the activity of the cyclooxygenase enzymes (COXs) and thereby affect the synthesis of prostaglandin signalling molecules, which are involved in a wide range of physiological processes beyond inflammation[8].

COXs catalyze the rate-limiting step in the production of prostaglandins (PG), bioactive compounds involved in processes such as fever and sensitivity to pain, and are the target of NSAIDs[9]. In mammals, the two COXs genes encode a constitutive isoenzyme (COX-1) and an inducible isoenzyme (COX-2); both of which are of significant pharmacological importance[9]. COX-1 is constitutively expressed in the majority of tissues and is associated with housekeeping functions such as vascular homeostasis and platelet aggregation[10]. COX-2, which is highly inducible and almost undetectable under normal physiological conditions, is readily induced in response to mitogens, tumor promoters, cytokines, growth factors, stress-inducing agents promoting inflammatory reactions, and tumor development[11]. COX-2 overexpression was found in a large proportion of GC tissues and was significantly associated with advanced tumor stage, *H. pylori* infection and lymph node metastasis[12]. COX-2 may also play a role in gastric carcinogenesis, which was associated with inhibition of apoptosis, increased metastasis potential and neoangiogenesis[13-16]. The expression of COX-2 is regulated by a complex signal transduction pathway in which many nuclear proteins interact with the COX-2 promoter region and play a decisive role in gene transcription[17]. Therefore, single nucleotide polymorphisms (SNPs) in the COX-2 promoter may have a great impact on gene transcriptional activity by altering the binding capability with certain nuclear proteins, resulting in inter-individual variability in susceptibility to cancer[18].

Systematic review can be a resourceful tool in detecting an association that could otherwise remain masked in the sample size studies, especially in those evaluating rare allele frequency polymorphisms[19]. The aim of this meta-analysis was to investigate the association of the COX-2 polymorphisms with susceptibility to GC by conducting a meta-analysis from all eligible case-control studies published to date.

MATERIALS AND METHODS

Search strategy and identification of studies

Medline, EMBASE and CBMdisc databases search were performed to retrieve papers linking COX-2 polymorphisms and susceptibility to GC available online by April 2010 without language restrictions, using the following query: [COX 2 OR COX-2 OR COX2 OR PEGS2 OR PEGS-2 OR "Cyclooxygenase 2" (MeSH)] AND [polymorphism OR polymorphisms OR "Polymorphism, Genetic" (MeSH) OR "Polymorphism, Single Nucleotide" (MeSH)] AND [gastric cancer OR "Stomach Neoplasms" (MeSH)]. The reference lists of major textbooks, review articles, and of all the included articles identified by the search were then individually searched to find other potentially eligible studies.

Inclusion criteria

To be eligible for inclusion in this meta-analysis, the following criteria were established: (1) the study must include a case-control study that addressed GC patients and normal or benign gastric diseases controls; (2) the study must have evaluated the COX-2 polymorphisms and susceptibility to GC; and (3) the study must have included sufficient data for extraction.

Exclusion criteria

Studies were excluded from consideration if: (1) the study was based on family data or incomplete raw data; (2) the study did not have the outcomes of comparison reported or it was not possible to determine them; or (3) the study contained a smaller sample size (number of cases < 30) and overlapped others.

Data extraction and quality assessment

Using a standardized form, data from published studies were extracted independently by two investigators (Liu JL and Liang Y) to populate the necessary information. From each of the included articles the following information was extracted: first author, year of publication, country, ethnicity, study design, source of cases, sample size, histological type, polymorphisms of genes, histopathological confirmations and evidence of Hardy-Weinberg equilibrium (HWE).

The quality of papers was also independently assessed by two researchers (Liu JL and Xing LL) based on the STROBE quality score systems[20]. 39 items relevant to the quality appraisal were used for assessment, scores ranged from 0 (lowest) to 50 (highest)[21]. Main items for quality assessment included: title and abstract, introduction, methods, study design, setting, participants, variables of interest, bias, HWE, sample size, statistical methods, funding and disclosure statement, internal validity, descriptive data, outcome data, main results, discussion, limitations, generality and interpretation[20,21].

Statistical analysis

Individual or pooled odds ratio (OR) and 95% confidence interval (CI) were calculated for each study using Review Manager version 5.0.23 software (provided by The Cochrane Collaboration, Oxford, England)[22]. Between-study heterogeneity was estimated using the χ^2-based Q statistic[23]. Heterogeneity was considered statistically

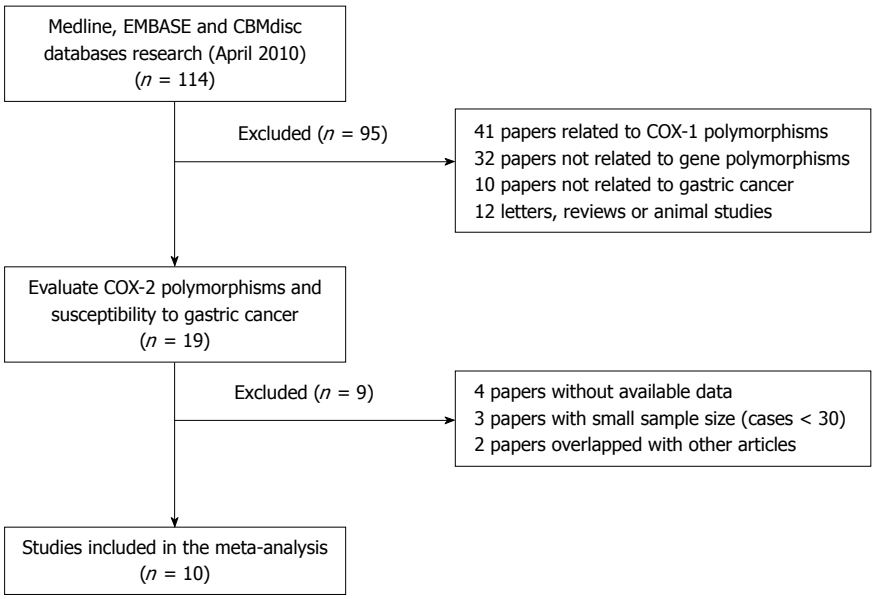

Figure 1 Flow chart showing study selection procedure. COX: Cyclooxygenases.

significant when $P_{\text{heterogeneity}} < 0.01$ or $I^2 > 50\%$. If heterogeneity existed, data was analyzed using a random effects model. In the absence of heterogeneity, a fixed effects model was used. Sources of heterogeneity were appraised by subgroup stratification analysis, based on several study characteristics, such as ethnicity and source of control individuals (population or hospital based). The funnel plot method was used to assess the possible presence of publication bias[24].

Before the effect estimation of the several COX-2 polymorphisms in gastric carcinogenesis, the HWE was assessed for all the polymorphisms in each study. A χ^2 test was performed to examine HWE when genotype data was available. If HWE disequilibrium existed ($P < 0.05$), or it was impossible to evaluate this equilibrium, sensitivity analysis was performed.

RESULTS

Search results

The search strategy retrieved 114 potentially relevant papers (52 in Medline, 35 in Embase, 27 in CBMdisc). There were 10 studies included in this meta-analysis. One-hundred and four studies were subjected to a full text review and excluded according to the selection criteria stated above. The flow chart of study selection is summarized in Figure 1.

Study characteristics and quality

In total, 3074 GC cases from 10 case-control studies were included in the meta-analysis. All studies were considered as case-control studies. Five ethnicities were addressed: two studies focused on Caucasian populations[25,26], five on Asian populations[27-31], one on a Hispanic population[32], one on a Dutch population[18] and one on an Indian population[33]. Eight studies[18,25,26,28-31,33] used hospital-based cases and controls and two studies[27,32] described a population-based design. Cases and controls of four studies[25,27,30,32] were mainly defined through endoscopic multiple biopsies procedures and three studies[18,31,33] through endoscopy, with histological assessment. However, there were three studies[26,28,29] that did not mention the method of confirmation. The characteristics and methodological quality of all studies are summarized in Table 1.

The characteristics of COX-2 polymorphisms in gastric cancer

Eleven COX-2 polymorphisms were addressed in gastric carcinogenesis: -765G>C, -1195G>A, Gly587Arg(G>A), 1290A>G, 8473T>C, IVS5-275T>G, IVS7+111T>C, V102V, *429T>C, *2430C>T, 587codonG>A. The genotype distribution of COX-2 polymorphisms and the variant allele frequency are described in Table 2. A HWE test was performed on all included studies, all of them showed in HWE ($P > 0.05$).

Association between COX-2 polymorphism and gastric cancer

Meta-analysis of eight studies identified a significant association between the -765C allele and susceptibility to GC (OR = 1.71, 95% CI: 1.01-2.90, $P = 0.05$) using the random effects model ($P_{\text{heterogeneity}} < 0.00001$, $I^2 = 89\%$). The -1195G>A COX2 polymorphism analysis revealed that the -1195A allele was also a risk factor for susceptibility to GC (OR = 1.58, 95% CI: 1.05-2.38, $P = 0.03$) using the random effects model ($P_{\text{heterogeneity}} = 0.04$, $I^2 = 70\%$). The -1290A>G analysis showed that the -1290G allele was a risk factor for GC (OR = 1.55, 95% CI: 1.01-2.39, $P = 0.05$). The *429T>C analysis also revealed that a significant association between the *429T allele and susceptibility to GC (OR = 0.74, 95% CI: 0.59-0.95, $P = 0.02$) using the fixed effects model ($P_{\text{heterogeneity}} = 0.42$, I^2

Table 1 Characteristics and quality assessment of 10 included case-control studies

Study	Yr	Country	Ethnicity	Polymorphisms of COX-2	No. of participants	Quality scores
Pereira *et al*[25]	2006	Portugal	Caucasians	-765G>C	73[a]/210[c]	33
Liu *et al*[27]	2006	China	Asians	-765G>C, -1195G>A, Gly587Arg(G>A)	248[a]/427[c]	33
Zhang *et al*[28]	2006	China	Asians	-765G>C, -1195G>A, -1290A>G	323[a]/646[c]	28
Jiang *et al*[29]	2007	China	Asians	-1195G>A, 8473T>C	254[a]/304[c]	36
Hou *et al*[26]	2007	Poland	Caucasians	-765G>C, IVS5-275T>G, IVS7+111T>C, V102V(G>C), *429T>C, *2430C>T	464[a]/480[c]	33
Canzian *et al*[32]	2008	Venezuela	Hispanics	IVS5-275T>G, V102V, *429T>C	1169[a]/1863[b]	39
Zhu *et al*[30]	2008	China	Asians	-765G>C, 587codonG>A	140[a]/125[c]	37
Saxena *et al*[33]	2008	India	Indians	-765G>C	62[a]/241[d]	34
Sitarz *et al*[18]	2008	Netherlands	Dutch	-765G>C	241[a]/100[c]	31
Tang *et al*[31]	2009	China	Asians	-765G>C	100[a]/105[c]	29

[a]Gastric cancer; [b]Precancerous gastric lesions; [c]Healthy controls; [d]Benign gastric diseases. COX-2: Cyclooxygenases-2.

Table 2 The genotype distribution of COX-2 polymorphisms and the variant allele frequency *n* (%)

Polymorphism	Study	Cases		Controls		Variant allele frequency	OR (95% CI)
		n	Variant allele carriers	*n*	Variant allele carriers		
-765G>C	Pereira *et al*[25]	73	37 (51)	210	80 (38)	0.22	1.67 (0.98-2.86)
	Liu *et al*[27]	388	42 (11)	427	43 (10)	0.05	1.08 (0.69-1.70)
	Zhang *et al*[28]	323	35 (11)	646	26 (4)	0.02	2.90 (1.71-4.91)
	Hou *et al*[26]	290	80 (28)	409	121 (30)	0.16	0.91 (0.65-1.27)
	Zhu *et al*[30]	140	38 (27)	125	20 (26)	0.06	1.96 (1.07-3.59)
	Saxena *et al*[33]	62	48 (77)	241	70 (29)	0.16	8.38 (4.34-16.16)
	Sitarz *et al*[18]	241	65 (27)	100	41 (41)	0.25	0.53 (0.33-0.87)
	Tang *et al*[31]	100	45 (45)	105	29 (28)	0.06	2.14 (1.20-3.84)
							Pooled OR = 1.71 (1.01-2.90)
-1195G/A	Liu *et al*[27]	389	316 (81)	427	322 (75)	0.50	1.41 (1.01-1.98)
	Zhang *et al*[28]	323	291 (90)	646	510 (79)	0.52	2.43 (1.61-3.66)
	Jiang *et al*[29]	254	206 (81)	304	242 (80)	0.26	1.17 (0.77-1.77)
							Pooled OR = 1.58 (1.05-2.38)
Gly587Arg (G>A)	Liu *et al*[27]	386	18 (15)	407	23 (6)	0.03	0.82 (0.43-1.54)
-1290A>G	Zhang *et al*[28]	323	40 (12)	646	54 (8)	0.04	1.55 (1.01-2.39)
8473T/C	Jiang *et al*[29]	254	95 (37)	304	105 (34)	0.11	1.13 (0.80-1.60)
IVS5-275T>G	Hou *et al*[26]	311	93 (30)	421	123 (29)	0.16	1.03 (0.75-1.42)
	Canzian *et al*[32]	108	36 (33)	1003	392 (39)	0.22	0.78 (0.51-1.19)
							Pooled OR = 0.93 (0.71-1.21)
IVS7+111T>C	Hou *et al*[26]	299	78 (26)	421	114 (27)	0.15	0.95 (0.68-1.33)
V102V	Hou *et al*[26]	302	72 (24)	410	125 (30)	0.16	0.71 (0.51-1.00)
	Canzian *et al*[32]	115	32 (28)	1053	214 (20)	0.11	1.51 (0.98-2.3)
							Pooled OR = 1.03 (0.49-2.14)
*429T>C	Hou *et al*[26]	304	167 (55)	165 (40)	251 (60)	0.36	0.80 (0.59-1.08)
	Canzian *et al*[32]	53 (49)	56 (51)	398 (38)	644 (62)	0.38	0.65 (0.44-0.97)
							Pooled OR = 0.74 (0.59-0.95)
*2430C >T	Hou *et al*[26]	289 (94)	19 (6)	399 (98)	10 (2)	0.01	2.62 (1.20-5.73)
587codonG>A	Zhu *et al*[30]	121 (86)	19 (14)	112 (90)	13 (10)	0.06	1.35 (0.64-2.87)

n: No. of patients; OR: odds ratio; 95% CI: 95% confidence interval; COX-2: Cyclooxygenases-2.

= 0%). A significant association with the susceptibility to GC was also determined in the *2430T allele analysis (OR = 2.62, 95% CI: 1.20-5.73, P = 0.02) (Figure 2). However, the Gly587Arg(G>A), 8473T>C, IVS5-275T>G, IVS7+111T>C, V102V and 587codonG>A polymorphisms showed no association with the susceptibility to GC (OR = 0.82, 95% CI: 0.43-1.54, P = 0.53; OR = 1.13, 95% CI: 0.80-1.60, P = 0.48; OR = 0.93, 95% CI: 0.71-1.21, P = 0.58; OR = 0.95, 95% CI: 0.68-1.33, P = 0.77; OR = 1.03, 95% CI: 0.49-2.14, P = 0.95 and OR = 1.35, 95% CI: 0.64-2.87, P = 0.43, respectively).

Subgroup analysis was performed on -765G>C polymorphism by ethnicity, such as Caucasian, Asian, Indian and Dutch. Results showed no significant association between the -765C allele and susceptibility to GC in Caucasians (OR = 1.19, 95% CI: 0.65-2.15, P = 0.58), while an obviously significant association was determined in Asians (OR = 1.87, 95% CI: 1.19-2.94, P = 0.006) and Indians (OR = 8.38, 95% CI: 4.34-16.16, P < 0.00001). However, the results showed that the -765C allele was a protective factor for GC in Dutch (OR = 0.53, 95% CI: 0.33-0.87, P = 0.01) (Figure 3). Sensitivity analysis was performed after excluding studies conducted by Saxena *et al*[33] and Sitarz *et al*[18], because of the anomalous OR and rare ethnicity.

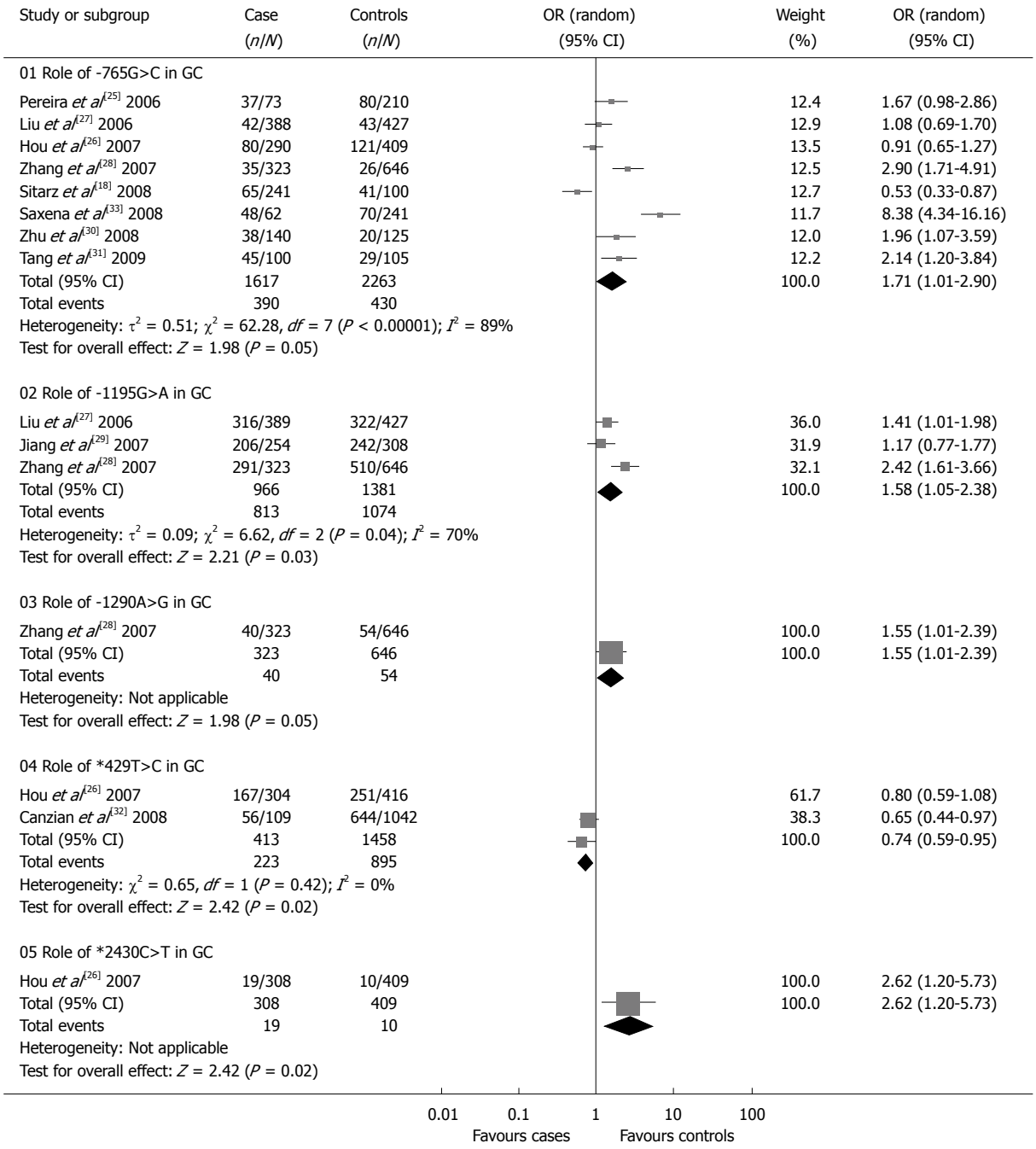

Figure 2 Meta-analysis of the association between cyclooxygenases-2 polymorphisms and susceptibility to gastric cancer.

No significant differences were observed between before and after results (OR = 1.59, 95% CI: 1.08-2.35, $P = 0.02$) (Figure 4).

Publication bias

The publication bias of the meta-analysis on the association between COX-2 and GC was detected by the funnel plot on -765G>C. The graphical funnel plot of 8 studies of -765G>C polymorphism appeared to be asymmetrical (Figure 5). Publication bias might occur if smaller studies showed no significant results remain unpublished, leading to an asymmetrical appearance of the funnel plot with a gap at the bottom of the graph.

DISCUSSION

Evidence suggests that COX-2 plays an important role in the carcinogenesis pathway, such as in the inhibition of apoptosis, tumor growth, angiogenesis, invasion and metastasis[34-36]. The specific function of COX-2 in the formation of prostaglandins makes it a strong candidate for increasing susceptibility to common cancers such as GC, colorectal cancer, lung cancer and other cancers[37]. As is known, genetic polymorphisms altering the level of protein expressed would be anticipated to have a substantial influence on disease activity. Several polymorphisms in COX-2 have been reported previously, although some

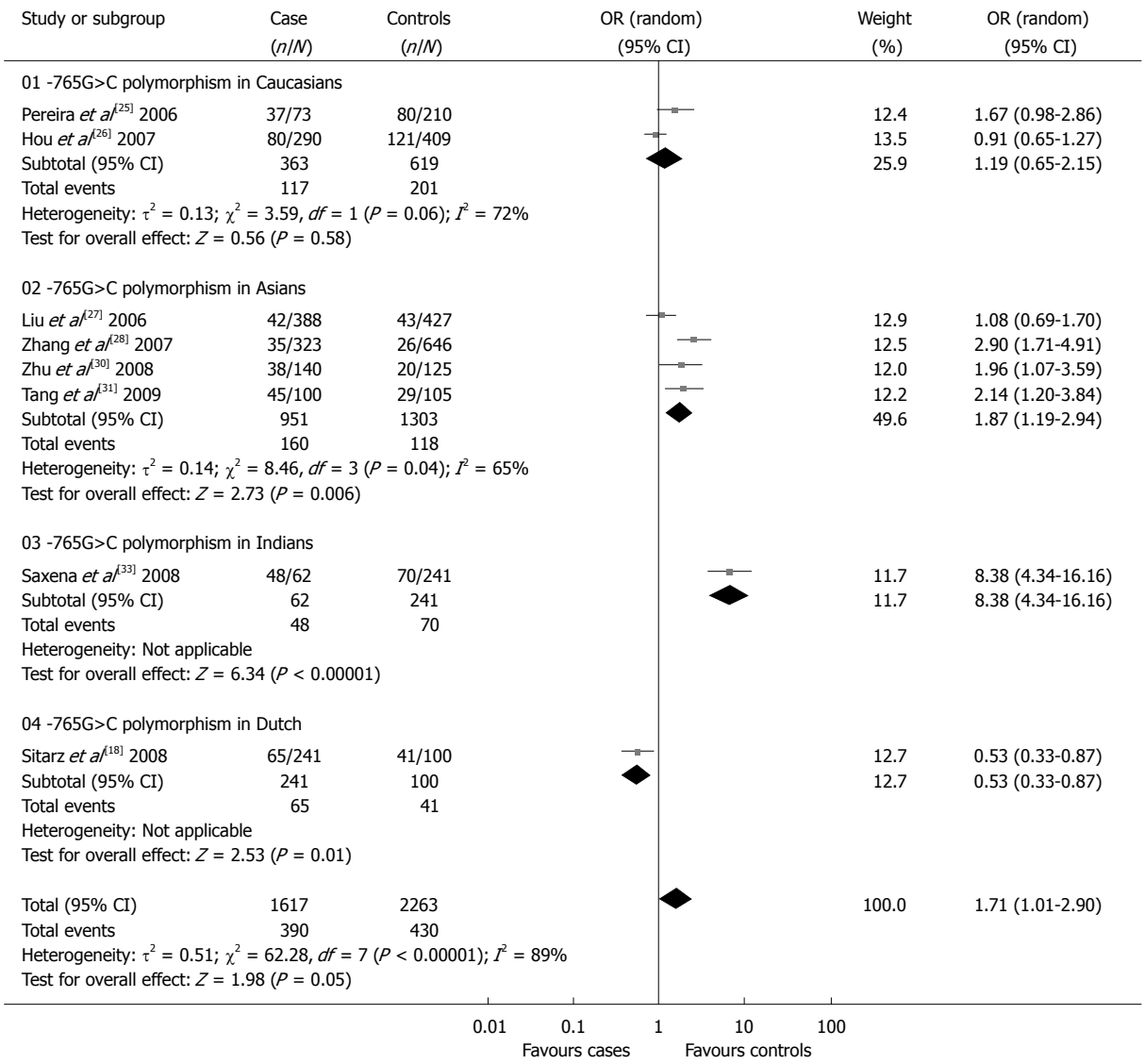

Figure 3 Subgroup analysis of -765G>C polymorphism by ethnicity.

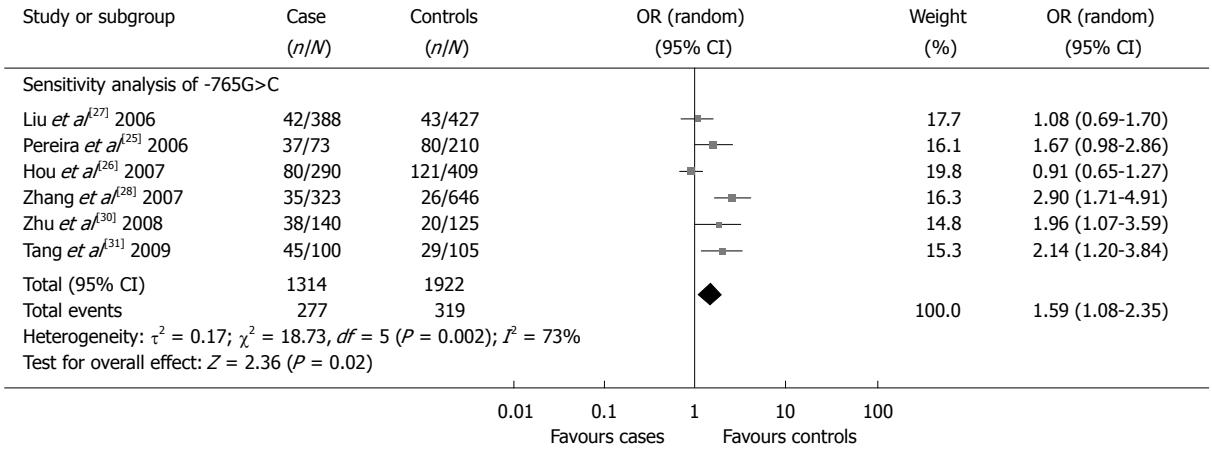

Figure 4 Sensitivity analysis of -765G>C polymorphism.

of these polymorphisms are not functionally significant or associated with susceptibility to cancer[21]. There were also many polymorphisms of COX-2 gene which were determined to have a significant association with the susceptibility to GC[21,38].

Our meta-analysis quantitatively assessed the asso-

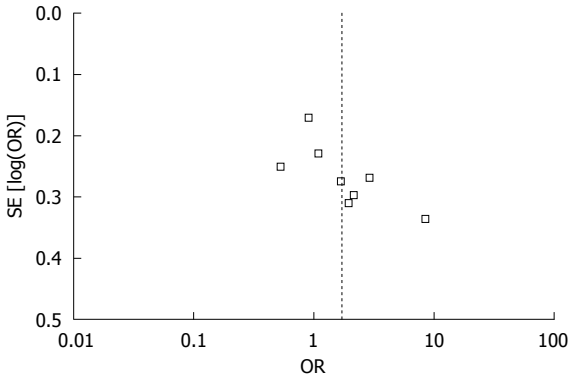

Figure 5 Funnel plots to explore publication bias.

ciation between COX-2 polymorphisms with the susceptibility to GC. Finally, 10 case-control studies were included and assessed. In this meta-analysis, 11 COX-2 polymorphisms were addressed and evaluated in gastric carcinogenesis: -765G>C, -1195G/A, Gly587Arg(G>A), 1290A>G, 8473T>C, IVS5-275T>G, IVS7+111T>C, V102V, *429T>C, *2430C>T, 587codonG>A. -765G>C, the most common polymorphism of the COX-2 gene and investigated in eight studies, revealed an increased risk behavior associated with gastric carcinogenesis in the normal population (OR = 1.71, 95% CI: 1.01-2.90, $P = 0.05$). As strong heterogeneity was reported, an ethnicity-based subgroup analysis was performed. The results showed that no association between the -765C allele and the susceptibility to GC in Caucasians, but an obviously significant association was determined in Asians (OR = 1.87, 95% CI: 1.19-2.94, $P = 0.006$) and Indians (OR = 8.38, 95% CI: 4.34-16.16, $P < 0.00001$). Interestingly, the -765C allele was a protective factor for GC in Dutch (OR = 0.53, 95% CI: 0.33-0.87, $P = 0.01$). Three studies discussed the association of the -1195G>A polymorphism and the susceptibility to GC. The results of the pooled analysis indicated that the -1195A allele was a risk factor for susceptibility to GC (OR = 1.58, 95% CI: 1.05-2.38, $P = 0.03$). In the study carried out by Zhang *et al*[28], the increased susceptibility was higher in individuals that were -1290G allele carriers (OR = 1.55, 95% CI: 1.01-2.39, $P = 0.05$). Unlike most of the COX-2 polymorphisms addressed, the *429C allele seems to play a protective role in GC development. However, the *429T allele was a risk factor for susceptibility to GC (OR = 0.74, 95% CI: 0.59-0.95, $P = 0.02$). Another study worth mentioning is the one by Hou *et al*[26], where we observed an increased risk of GC in Caucasians population with the *2430T allele (OR = 2.62, 95% CI: 1.20-5.73, $P = 0.02$). Unfortunately, no statistically significant results were observed in the Gly587Arg(G>A), 8473T>C, IVS5-275T>G, IVS7+111T>C, V102V and 587codonG>A COX-2 polymorphisms in gastric carcinogenesis. Therefore, in further functional studies, future investigations should focus on the -765G>C, -1195G>A, -1290A>G, *429T>C, and *2430C>T COX-2 polymorphisms and their molecular mechanism involving gastric carcinogenesis.

There were some limitations in our meta-analysis. Firstly, because of incomplete raw data or publication limitations, some relevant studies could not be included in our analysis. Secondly, we were not able to address the sources of heterogeneity that existed among studies for most polymorphisms. However, we could not perform subgroup stratification analysis for the limited number of published studies. Thirdly, the lack of genotype frequency information provided by some published studies did not allow the estimation of the best genetic model of inheritance to follow. Although we actively contacted the authors, they did not provide a comprehensive set of data. In addition, the small sample size available was not ideal for detecting small genetic effects. Finally, our systematic review was based on unadjusted data, as the genotype information stratified for the main confounding variables was not available in the original papers and also the confounding factors addressed across the different studies were variable.

In conclusion, our meta-analysis of 10 case-control studies demonstrated an association between the -765C, -1195A, -1290G, *2430T alleles and *429TT genotype of COX-2 polymorphisms and GC. In addition, all of these findings suggested that ethnicity was the main sources of heterogeneity, and different ethnicities with COX-2 polymorphisms had varying susceptibility to GC. From the analysis, we also concluded that the Gly587Arg(G>A), 8473T>C, IVS5-275T>G, IVS7+111T>C, V102V and 587codonG>A polymorphisms showed no association with susceptibility to GC. As few studies are available in this field and current evidence remains limited, the necessity should be emphasized to conduct large studies with an adequate methodological quality, properly controlling for possible confounds in order to obtain valid results.

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COMMENTS

Background

Accumulated evidence indicates that the cyclooxygenases-2 (COX-2) play an important role in gastric carcinogenesis, which was associated with inhibition of apoptosis, increased metastasis potential and neoangiogenesis. To investigate the association of COX-2 polymorphisms with susceptibility to gastric cancer (GC), the authors carried out a meta-analysis of all related case-control studies.

Research frontiers

Much attention has been paid to the potential role of COX-2 in gastric carcinogenesis. Some of them have been trying to confirm the definite relationship between COX-2 polymorphisms and susceptibility to GC, and meanwhile, others attempt to uncover underlying mechanisms.

Innovations and breakthroughs

The current study demonstrated that the -765C, -1195A, -1290G, *2430T alleles and *429TT genotype of COX-2 polymorphisms have significant association regarding the susceptibility to GC.

Applications

Meta-analysis suggests that the -765C, -1195A, -1290G, *2430T alleles and *429TT genotype of COX-2 polymorphisms were statistically significant risk

factors for GC. These genetic profiles may enable clinicians to select individuals for early diagnosis strategies, diverse management schedules such as the follow-up of patients with GC, or even to propose selective COX-2 inhibitors or nonspecific COX inhibitors in patients with precancerous lesions.

Terminology

Meta-analysis is a statistical tool in detecting an association that could otherwise remain masked in the sample size studies, especially in those evaluating rare allele frequency polymorphisms.

Peer review

This is an interesting and good paper, the described analysis has been performed with precision and accuracy.

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CASE REPORT

Sirolimus plus sorafenib in treating HCC recurrence after liver transplantation: A case report

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Abstract

A case of hepatocellular carcinoma (HCC) with pulmonary recurrence after liver transplantation for HCC is presented in this report. The patient showed disease progression on sorafenib therapy demonstrated by computed tomography scans as well as serial serum α-fetoprotein (AFP) elevation. After his immunosuppression therapy was successfully transitioned to sirolimus and a continuation of sorafenib, he achieved partial remission based on RECIST criteria and normalization of AFP. Mammalian target of rapamycin inhibitors including sirolimus alone or in conjunction with sorafenib may be useful in the treatment of post transplant HCC.

Key words: Hepatocellular carcinoma; Liver transplantation; Mammalian target of rapamycin inhibitor; Sirolimus; Sorafenib

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem with a rising incidence in Western countries[1,2]. It is the third leading cause of cancer-related deaths worldwide, the seventh in the United States, and also the most common cause of death in patients with liver cirrhosis. The trend parallels an increase in advanced hepatitis C virus (HCV) related liver disease. The prevalence is much higher in developing countries and is associated with a significant morbidity and mortality. The treatment of HCC remains challenging due to the comorbidities with hepatitis B or C virus infection combined with decompensated liver cirrhosis. Only about 30% of patients with HCC are eligible for potentially curative treatment modalities such as liver resection, liver transplantation, or local ablation[3]. Conventional cytotoxic chemotherapeutic agents have been proven ineffective for patients with HCC[4]. Based on the SHARP trial[5], the Raf/vascular endothelial growth factor receptor (VEGF)/platelet derived growth factor receptor inhibitor, sorafenib, has increased the overall survival rate in patients with advanced HCC. The study was carried out in a highly selected population of HCC patients with liver function classified as Child-Pugh A and a small percentage of patients classified as Child-Pugh B. Orthotopic liver transplantation (OLT) for HCC has been highly successful with a low tumor recurrence rate in selected patient

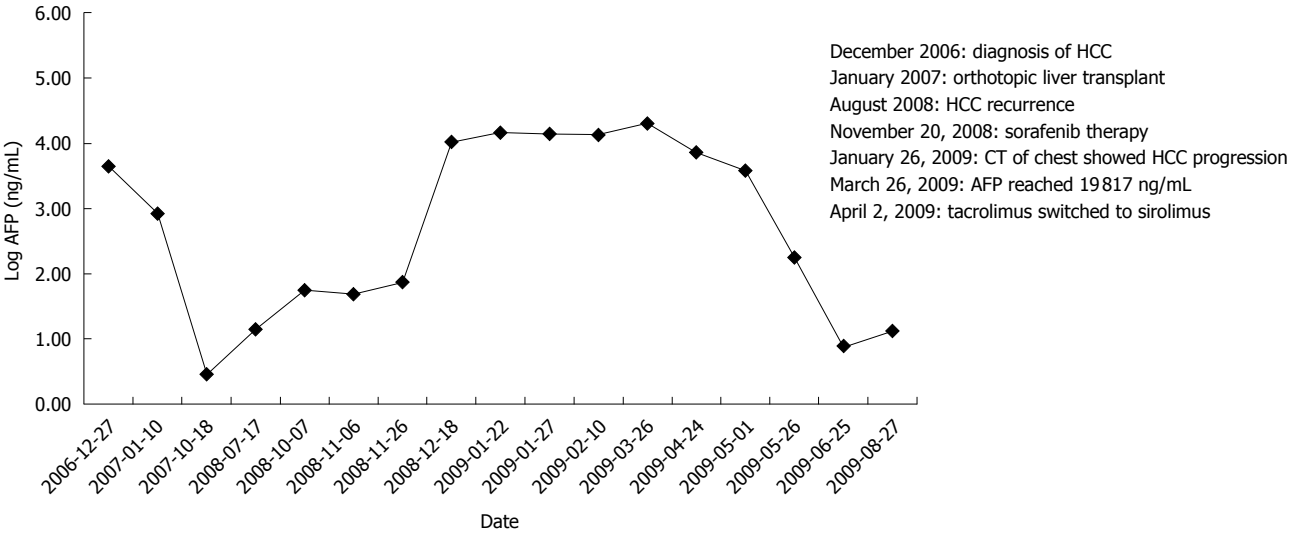

Figure 1 Serum α-fetoprotein level measured as related to the treatment history. Serum α-fetoprotein (AFP) level was trending up after sorafenib therapy but normalized after sirolimus was initiated. Events are outlined in the Figure. CT: Computed tomography; HCC: Hepatocellular carcinoma.

populations[6]. Tumor recurrence post OLT is the major obstacle in preventing successful liver transplantation in patients with HCC. Therefore, it is important to develop strategies for preventing HCC recurrence post OLT. Here, we present a case of HCC recurrence post OLT that was treated with sorafenib in addition to switching from tacrolimus to sirolimus as an immunosuppressant.

CASE REPORT

A 60-year-old male with a history of hepatitis C virus infection and liver cirrhosis was found to have several liver lesions consistent with HCC with an elevated α-fetoprotein (AFP) level on routine surveillance in December 2006. Based on his typical images and serum AFP level, the patient was diagnosed as HCC. He underwent an uneventful OLT at our liver transplant center in January 2007. Pathology showed multi-focal HCC with 4 lesions (3 lesions preoperatively), the largest measuring 5.0 cm × 3.0 cm × 2.9 cm. The tumors were moderately differentiated with vascular invasion and the pathologic staging was pT2N0Mx. The patient was given tacrolimus 4 mg twice a day for immunosuppression with serum tacrolimus levels at a range of 5-15 ng/mL after liver transplantation. HCV hepatitis recurrence was diagnosed by liver biopsy and serology in February 2008 when the patient had laboratory evidence of transaminase elevation. He continued on tacrolimus with no anti-HCV therapy and was followed up by the liver transplant team. In August 2008, he was admitted to the hospital for renal failure and hyperkalemia suspicious for tacrolimus toxicity with an elevated tacrolimus level at 16 ng/mL. His creatine level was 1.7 mg/dL. His tacrolimus dose was reduced to 2 mg twice a day orally and his tacrolimus target levels were at a low normal therapeutic range. During hospitalization, he was found to have new pulmonary nodules on a computed tomography (CT) scan. A biopsy confirmed those lesions to be metastatic HCC with its morphology similar to his original HCC. Due to bilateral pulmonary metastasis, the patient was not a candidate for surgery. He was referred to the medical oncology clinic in October 2008 for treatment. The recommendation was sorafenib therapy for his recurrent metastatic HCC after liver transplantation. Four weeks after he started on oral sorafenib at a dose of 400 mg twice a day from November 20, 2008, he developed the adverse effects of painful hand/foot syndrome. The dose of sorafenib was reduced to 200 mg orally twice a day on December 18, 2008. He tolerated sorafenib at 200 mg orally twice daily very well with less chest wall pain and resolution of hand/foot syndrome. His CT scan on January 26, 2008 showed progression of the disease based on RECIST criteria. Since the patient had improvement in symptoms, he wanted to continue taking sorafenib at 200 mg orally twice daily. Unfortunately, his AFP continued rising and reached a peak level of 19817 ng/mL on March 26, 2009 (Figure 1). The metastatic tumors in his lungs were also increasing in size based on a CT scan performed on January 26, 2009. He was advised to consider systemic chemotherapy due to his excellent performance status and good graft liver function, but there was no other evidence based treatment available for him. He refused systemic chemotherapy after the literature of chemotherapy in HCC was reviewed with him. He was not eligible for an experimental trial since he was on an immunosuppressant and had active hepatitis C virus infection.

Given the positive preclinical data on mammalian target of rapamycin (mTOR) inhibitors in HCC as well as the potential benefit of sirolimus for HCC patients requiring immunosuppression, his immunosuppressant was switched from tacrolimus to sirolimus at 2 mg orally once daily for a week after the visit on March 26, 2009. He continued taking sorafenib at 200 mg orally twice a day against advice from his physician because he believed that it helped him symptomatically. He

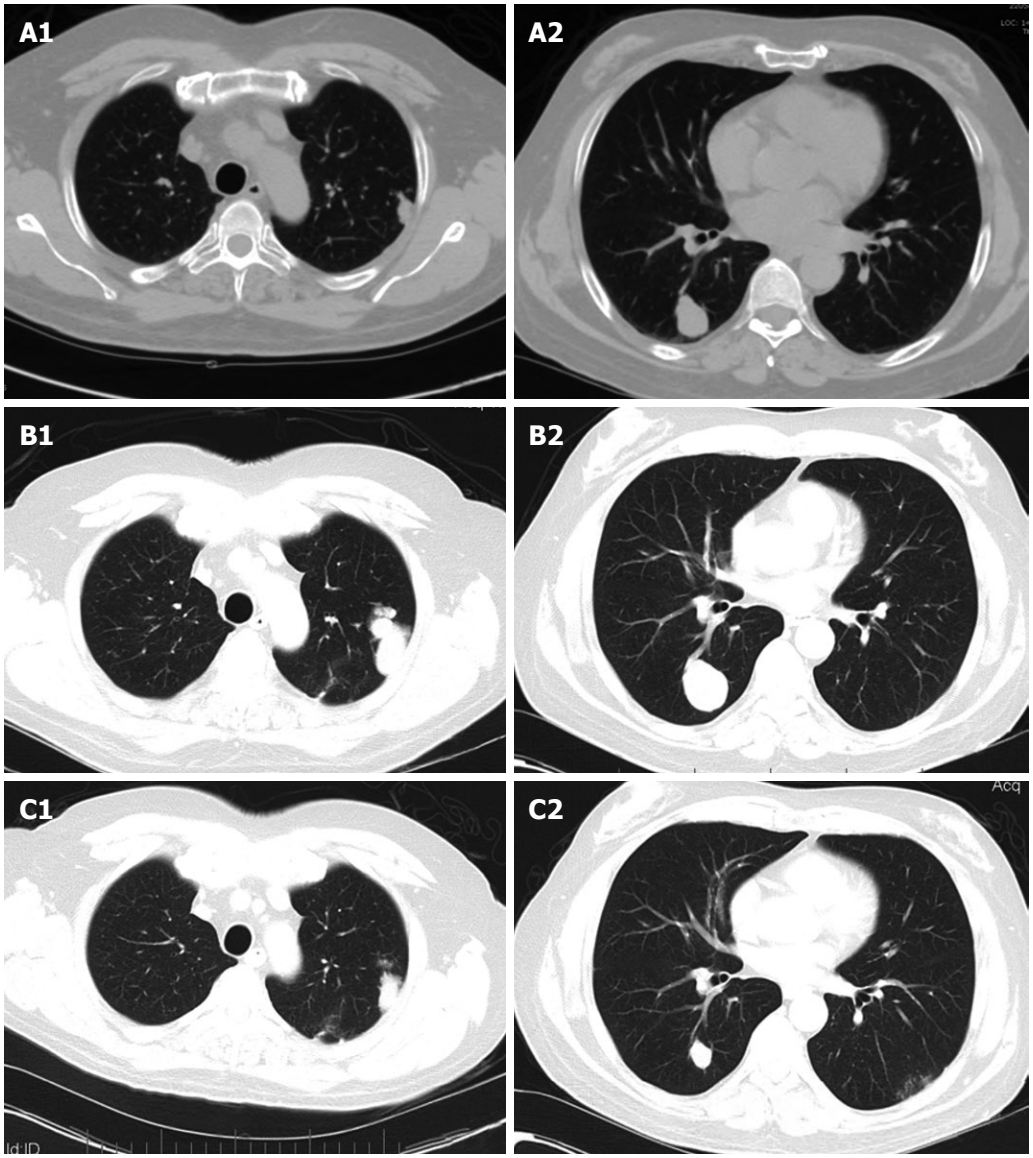

Figure 2 Computed tomographys of chest at different time points of the treatment. The lung lesions were increased in size after sorafenib therapy but significantly reduced in size after sirolimus was introduced in combination with sorafenib. The dates were August 6, 2008 (A), January 26, 2009 (B) and August 26, 2009 (C).

tolerated sirolimus at 2 mg orally daily with no obvious toxicity, and a desirable serum level (4-12 ng/mL) of sirolimus was achieved shortly after sirolimus therapy was initiated. His AFP dropped to normal limits and the CT showed a partial response (Figure 2). The dates for the representative images shown in Figure 2 were August 6, 2008, January 26, 2009 and August 26, 2009. There was no evidence of biochemical or radiographical progression when he was seen in the clinic on February 4, 2010.

DISCUSSION

The treatment algorithm for HCC is based on functional status, liver functional reserve and stage of the tumor[3]. Surgical resection of HCC can be achieved in patients with a tumor that is amenable to surgical resection and has a good liver functional reserve. For smaller tumors, local therapy with radiofrequency ablation is used. OLT in selected patients is the most desirable modality because it cures both liver cirrhosis and HCC. Tumor recurrence is a cause of transplant failure in this population of patients. Adjuvant therapy for HCC post liver transplantation is an active research area but currently there is no evidence that supports the practice.

The treatment of recurrent HCC after liver transplantation is a great challenge in clinical practice. The use of immunosuppressant and liver transplantation are usually exclusion criteria for participation in clinical trials. The use of sorafenib in this setting is an extrapolation of data and by no means evidence-based care.

The most commonly used immunosuppressant after liver transplantation is a calcineurin inhibitor. In contrast, sirolimus, a mTOR inhibitor has also been used successfully for immunosuppression after liver transplantation[7].

In comparison to tacrolimus as an immunosuppressant, the advantages of sirolimus include less nephrotoxicity and potential antineoplastic properties. This makes it suitable for patients with renal insufficiency or HCC. The drug development for mTOR inhibitors has generated several novel anti-cancer mTOR inhibitors which have been approved by the FDA for the treatment of renal cell carcinoma[8,9]. The use of sirolimus has been limited to immunosuppression in organ transplantation patients. There is an ongoing phase 3 clinical trial (SiLVER trial) studying the use of sirolimus as an immunosuppressant in liver transplantation for high risk HCC to reduce tumor recurrence (ClinicalTrials.gov Identifier: NCT00355862). Currently, there is no standard therapy for HCC recurrence post OLT. Sorafenib has been used empirically for relapsing HCC after OLT, but there are no data supporting the use of sorafenib in this setting. Our own experience with sorafenib for recurrent HCC post liver transplantation is disappointed. Preclinical and clinical data indicate that mTOR inhibitors have significant anti-tumor activity for HCC[10-13]. There was a case report of complete remission of lung metastases from HCC post liver transplantation under therapy with sirolimus and mycophenolate mofetil[14], and experience with the use of sirolimus as an immunosuppressant for patients with HCC post OLT.

The use of sirolimus as an immunosuppressant is a reasonable approach for patients with HCC post liver transplantation for HCC or for patients with relapsing HCC post liver transplantation. The PI3K/Akt/mTOR signal pathway is involved in multiple cellular functions including proliferation, differentiation, tumorigenesis, and apoptosis. In about 15%-41% HCC patients, activation of the mTOR pathway is reported[15-17] and implicated in metastasis, invasion and prediction of poor prognosis[10,18]. Blocking of the mTOR pathway confers combined anticancer and immunosuppressive properties. Therefore, mTOR inhibitor treatment for HCC is directed by the molecular biology of HCC. Multiple clinical trials have been carried out using an mTOR inhibitor as an anticancer agent for HCC. Experimental data indicate that tumor inhibition can be accomplished by mTOR inhibitors while protecting allografts against rejection. The role of mTOR inhibitors as an immunosuppressant in HCC patients post OLT can be further determined depending on data from the SiLIVER trial. The case presented in this report clearly suggests that an mTOR inhibitor, sirolimus, may have antitumor activity in this patient with advanced metastatic relapsing HCC post OLT. This conclusion is also supported by published preclinical data[12]. The patient was at a high risk of tumor recurrence based on the presence of multifocal liver lesions and vascular invasion. Molecular evaluation of tumor samples from patients with HCC may give us an insight about post surgical or post transplantation adjuvant therapy using either sorafenib, sirolimus or their combination. The hypothesis that sirolimus synergizes with the anti-tumor activity of sorafenib is supported by pre-clinical data[12], but whether this applies to the patient reported here is unknown. The tumor sample is being analyzed for Erk/MAPK pathway and PI3/Akt/mTOR pathway abnormalities. The fact that the patient did not derive any apparent benefit from more than 4 mo of sorafenib therapy alone after his tumor recurrence indicated that his HCC had inherent resistance to the Raf/VEGF/platelet derived growth factor receptor inhibitor, sorafenib. No reports are available on the delayed antitumor effects of sorafenib. Biomarkers that predict the effectiveness of mTOR inhibitor and/or sorafenib therapy and clinical trials to explore the effectiveness of sirolimus alone and in combination with sorafenib with molecular pathway correlation analysis are justified.

In conclusion, mTOR inhibitor, sirolimus, may have anti-tumor activity in HCC. Use of sirolimus as an immunosuppressant for HCC post OLT is being investigated, and its use in HCC alone or in combination with sorafenib warrants further investigation with biomarker correlation.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
San Francisco, CA, United States
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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 Pixudiarrhoea. *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]

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Personal author(s)

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

Chapter in a book (list all authors)

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

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

Conference proceedings

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

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

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

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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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Contents

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EDITORIAL

Interplay between inflammation, immune system and neuronal pathways: Effect on gastrointestinal motility

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Abstract

Sepsis is a systemic inflammatory response representing the leading cause of death in critically ill patients, mostly due to multiple organ failure. The gastrointestinal tract plays a pivotal role in the pathogenesis of sepsis-induced multiple organ failure through intestinal barrier dysfunction, bacterial translocation and ileus. In this review we address the role of the gastrointestinal tract, the mediators, cell types and transduction pathways involved, based on experimental data obtained from models of inflammation-induced ileus and (preliminary) clinical data. The complex interplay within the gastrointestinal wall between mast cells, residential macrophages and glial cells on the one hand, and neurons and smooth muscle cells on the other hand, involves intracellular signaling pathways, Toll-like receptors and a plethora of neuroactive substances such as nitric oxide, prostaglandins, cytokines, chemokines, growth factors, tryptases and hormones. Multidirectional signaling between the different components in the gastrointestinal wall, the spinal cord and central nervous system impacts inflammation and its consequences. We propose that novel therapeutic strategies should target inflammation on the one hand and gastrointestinal motility, gastrointestinal sensitivity and even pain signaling on the other hand, for instance by impeding afferent neuronal signaling, by activation of the vagal anti-inflammatory pathway or by the use of pharmacological agents such as ghrelin and ghrelin agonists or drugs interfering with the endocannabinoid system.

Key words: Sepsis; Ileus; Nitric oxide; Prostaglandins; Oxidative stress; Residential macrophages; Mast cells; Neurons; Afferent; Neuroimmunomodulation; Inflammation

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DEFINITION AND CLINICAL RELEVANCE OF SEPSIS

Sepsis originates from the Greek word sepsios meaning "rotten" or "putrid". Sepsis is defined as a systemic inflammatory response syndrome secondary to infection. It represents a leading cause of death in critically ill patients, mainly due to the development of organ dysfunction and tissue hypoperfusion[1-3]. The incidence of severe sepsis is still increasing and ranges from 11%-15% in intensive care unit (ICU) patients: 11.8 patients per 100 ICU admissions in an Australian and New Zealand population[4]; 14.6% in a French ICU population[5], 11% of all ICU admissions in the US[6] and 12% of ICU patients in Spain[7]. Although

the overall mortality rate among patients with sepsis is declining - related to general improvements in acute and intensive hospital care rather than specific sepsis-related therapy - the number of sepsis-related deaths still increases and ranges between 25%-60%[2,4-6,8]. The development of organ dysfunction is a major determinant of mortality and is influenced by the co-existence of chronic comorbidity[8].

According to the International Guidelines of Severe Sepsis 2008[3], the management of severe sepsis complicated with hypoperfusion and organ failure is based on initial resuscitation (first 6 h) with, as main goals, maintaining the mean arterial pressure above 65 mmHg, a CVP of 8-12 mmHg, a urinary output above 0.5 mL/kg per hour and a central venous oxygen saturation above 70%. Secondly, the source and type of infection needs to be established by obtaining appropriate cultures before antibiotic therapy is initiated. The use of fluid therapy, vasopressors (norepinephrine and dopamine as initial choice) and dobutamine as inotropic therapy is recommended[3]. The use of corticosteroids remains controversial in the management of sepsis and is advised only in refractory sepsis, as is the use of recombinant human activated protein C which should be reserved for patients with organ dysfunction and a high clinical risk of death[2,9].

During sepsis a complex interaction takes place between the infecting microorganism, the host immune response, inflammatory and coagulation responses[9]. Different mechanisms such as the innate immune system, the coagulation pathways, endothelial dysfunction, mitochondrial dysfunction and apoptosis are described and associated with severe sepsis[9]. A cross-talk takes place between different immune cells including macrophages, dendritic cells and CD4+ T cells, leading to either a proinflammatory or anti-inflammatory cytokine reaction[10]. Patients can thus present with either an exaggerated proinflammatory systemic inflammatory response (often described in the early phase) or rather a state of immunosuppression and even anergy in a later phase[10,11]. Generally accepted is the theory that cells of the innate immune system recognize microorganisms and initiate responses through pattern recognition receptors (PRRs), pathogen-associated molecular patterns (PAMPs) and Toll-like receptors (TLRs). The latter will result in activation of intracellular signal transduction pathways such as the activation of nuclear factor (NF)-κB and caspase-1[9,11]. On the other hand, an excess production of reactive oxygen and nitrogen species is described resulting in oxidative and nitrosative stress. Other pathogenic mechanisms leading to sepsis-related organ dysfunction are exacerbated coagulation, impaired anticoagulation and decreased fibrin removal, together with endothelial disturbances, mitochondrial dysfunction and apoptosis[9].

ROLE OF THE GASTROINTESTINAL TRACT IN SEPSIS

Hassoun *et al*[12] described in the early 2000s how the gastrointestinal tract might play a pivotal role in the pathogenesis of post-injury multiple organ failure. Gut hypoperfusion is an important inciting event in the pathogenesis of organ failure, whereas the reperfused gut is an early source of proinflammatory mediators. Ischemia and reperfusion in the gastrointestinal tract will activate a cascade of stress-sensitive protein kinases (MAPK, ERK, p38, JNKs) that converge on transcription factors regulating the expression of proinflammatory genes[12]. Important mechanisms playing a role in gastrointestinal dysfunction as a result of post-injury multiple organ failure are increased intestinal permeability, bacterial translocation and paralytic ileus[13]. Bacterial translocation is defined as the passage of both viable and non-viable microbes and microbial products such as endotoxins across the mucosal barrier[12], whereas ileus is defined as an inhibition of propulsive intestinal motility[14]. These mechanisms play an important role in the maintenance of multiple organ failure and secondary infections[12]. Frequently, the source of bacteria can be traced to the endogenous flora of the gastrointestinal tract[15]. Ileus predisposes to luminal accumulation and bacterial colonisation of the stomach and small intestine and therefore promotes bacterial translocation and pneumonia by aspiration of gastric contents. Ileus therefore plays a pivotal role in the occurrence and maintenance of infections in multiple organ failure[12]. The guidelines on the management of severe sepsis of 2008 concluded that prophylactic use of selective digestive tract decontamination in severe sepsis patients would be targeted towards preventing secondary ventilator-associated pneumonia[3]. There are, however, insufficient data available from severe sepsis patients to support global use of selective digestive tract decontamination.

The gastrointestinal tract therefore has a dual role in sepsis, being a target organ and a pathogenic player. It is now understood that the gut is not only a source of bacteria and endotoxins, but also a source of pro-inflammatory mediators and a cytokine-generating organ. These inflammatory mediators reach the circulation *via* the intestinal lymph[16,17]. Bacteria and endotoxins crossing the mucosal barrier further potentiate the gut inflammatory response, even when the bacteria and their products are trapped within the gastrointestinal wall or intestinal lymph nodes, not reaching the systemic circulation[16]. Studying the impact of experimentally-induced sepsis on gastrointestinal motility and its immunological modulation therefore merits further attention.

ANIMAL MODELS OF SEPSIS

Several animal models of sepsis exist, all with their advantages and disadvantages (Table 1)[18-20]. One of the main criticisms of the animal models is that the demonstrated benefits of therapeutic agents in animals are rarely translated into successful clinical trials, indicating the difficulty of mimicking the complex interaction between current illness, sepsis and supportive therapy in an animal model. The lack of supportive therapeutic interventions in animal models represents therefore an important caveat in the use of animal models. Also, the

Table 1 Overview of septic animal models displaying advantages and disadvantages (adapted from[18-20])

Endotoxin model
Advantages
Endotoxins play a significant role in the pathogenesis of sepsis
Simple model
Using sublethal doses, providing active resuscitation, using continuous infusion and the use of intraperitoneal injection are four measures reproducing more accurately the human situation
Lipopolysaccharides is stable (compared to the use of bacteria), therefore the model is more accurate and reproducible compared to the bacterial infection models
Disadvantages
Exaggerated release of host cytokines
Most of the time only Gram-negative sepsis
Single toxin does not mimic human sepsis
Therapies shown to be effective in animal models, failed in clinical trials
Rats are very resistant compared to humans
Lack of an infectious focus
Bacterial infection model
Advantages
Endotoxins play a significant role in the pathogenesis of sepsis
Reduction of the dose, increasing the infusion time, giving active resuscitation can prolong survival and render the model more comparable to the human situation
Disadvantages
Uncommon clinical occurrence
High doses of bacteria are needed
Significant interlaboratory variability
Survival is short
Serum cytokine responses are transient and exaggerated
Peritonitis model: cecal ligation puncture model
Advantages
Resemblance to clinical situation
Peritoneal contamination with a mixed flora
The cytokine response is comparable to human situation
Severity can be adjusted by increasing the needle puncture size or the number of punctures, delaying mortality over several days
Disadvantages
The model needs a surgical procedure that by itself may induce ileus
Difficult to control the magnitude of septic challenge
Variability within the cecal ligation puncture model

timing of most animal models is not comparable to the human situation as most animal models represent acute syndromes unlike sepsis in humans (hours to days in animal models *vs* days to weeks in humans)[20].

Animal models of sepsis are generally divided into 3 categories: endotoxin models, bacterial infection models and peritonitis models[18-20]. The major advantages and disadvantages of these models are described in Table 1. In the endotoxin model lipopolysaccharides (LPS) of bacteria are injected, while in the bacterial infection models the bacteria themselves are injected. Different peritonitis models are described, such as cecal ligation and puncture (CLP), implantation of a fibrin clot suspended with bacteria in the abdominal cavity or implantation of a colonic stent. These peritonitis models have as a major advantage the presence of a local infection focus and some authors consider the CLP model as the gold standard for sepsis research[20]. However, it is important to understand that this procedure requires a major surgical procedure which might have no effect in sepsis survival studies but strongly interferes with gastrointestinal motility because of the induction of postoperative ileus.

In the endotoxin model, LPS is injected intravenously or intraperitoneally. The choice of the animal (mouse, rat, guinea pig), and the strain and gender of the animal are all confounding parameters in these models, as well as the strain of bacteria or endotoxins used, the dose and the administration route. Studying gastrointestinal motility and its immunological modulation by administering a single intraperitoneal injection of endotoxin at a sublethal concentration represents an adequate model for experimental sepsis[13,21]. It has been shown previously in different animal species that a single dose of LPS alters gastrointestinal motility. By 1963, Turner *et al*[22] had already shown that endotoxins reduce water and food intake and gastric emptying in mice. We investigated the effect of a single intraperitoneal injection of LPS of *Escherichia coli* and showed a significant delay in gastric emptying and small intestinal transit[23-26]. In rats, endotoxins delay gastric emptying, increase small intestinal transit[27-31], and reduce jejunal spontaneous circular muscle activity[32,33]. In dogs, endotoxins delay gastric emptying and abolish intestinal migrating motor complexes[34-37]. In horses, a low dose of endotoxin was reported to disrupt the motility pattern and to decrease the cecal and colonic contractile activity[38,39]. Therefore, endotoxins are definitely able to induce gastrointestinal ileus, which we will now refer to as sepsis- or endotoxin-induced ileus.

On the other hand, ileus is often studied in a surgically-induced postoperative model. It is generally accepted that postoperative ileus is triggered by two different phases: an early neurogenic and a late inflammatory phase[40]. During the initial neuronal phase, inhibitory effects on motility are related to the activation of an inhibitory reflex pathway involving adrenergic, nitrergic and VIP-ergic neurons[41,42]. In the second phase, the activation of an inflammatory cascade plays a crucial role and is triggered by the handling of the intestines activating the cross-talk between the immune system, the autonomic nervous system and the muscle effector apparatus of the gastrointestinal wall[40].

Both the endotoxin-induced model and the postoperative ileus model accentuate the important role of inflammation in the development and maintenance of gastrointestinal ileus. It is therefore our opinion that the endotoxin-induced ileus model and the postoperative ileus model are both relevant in the study of inflammation-induced motility disturbances.

PATHOGENESIS OF INFLAMMATORY-MEDIATED ILEUS

Initial research focussed on the mediators that could be involved in the inflammation-induced impairment of gastrointestinal motility by a direct action on the intestinal smooth muscle cells. Later on, the focus was broadened in an attempt to clarify not only the mediators involved but also the cell types and the transduction pathways. The

main goals were to identify possible target molecules enabling the development of novel drugs for clinical use.

Mediators involved in the pathogenesis of inflammatory-mediated ileus

Nitric oxide (NO) was one of the first molecules postulated to play an important role in the pathogenesis of LPS-induced motility disturbances in rats and mice mainly mediated *via* the inducible isoform of NO synthase (iNOS)[23,27,29,30,32,43]. Several groups showed that blockade of NOS reverses the endotoxin-induced changes in gastrointestinal motility in different animal species by the use of selective or non-selective NOS blockers and iNOS knock-out mice[23,27,29,30,32]. Our group proved that this effect of NO, derived from iNOS, is mediated at least partially by activation of guanylyl cyclase in a murine endotoxic model[23,44]. Furthermore, we also found evidence of a role for NO-mediated oxidative stress mechanisms, indirectly *via* the use of the solvent DMSO which also has radical scavenging properties, and directly by the use of antioxidant molecules[23,24,45]. Treatment of mice with the antioxidant pegylated superoxide dismutase reversed the endotoxin-induced delay in gastric emptying and improved the delay in intestinal transit. This was associated with a decrease of iNOS-positive residential macrophages and a decrease of immunohistochemical staining for nitrotyrosine and 4-hydroxy-2-nonenal, markers for oxidative and nitrosative stress, in the gastric and ileal mucosa of LPS-treated mice[24]. In agreement with these results, we found that the antioxidant melatonin reversed the endotoxin-induced motility disturbances in mice through a reduction of intestinal lipid peroxidation, MAPK activation, NF-κB activation, iNOS transcription and expression, and nitrite production[45].

In addition, NO produced from neuronal NOS may be involved in sepsis-induced ileus. Quintana *et al*[46,47] showed synthesis of NO in postganglionic myenteric neurons during the early phase of endotoxemia (30 min after injection of LPS) in rats, as well as an increase in nNOS mRNA in the dorsal vagal complex of the brainstem 2 h after administration of LPS.

Prostaglandins are also postulated to play an important role in the pathogenesis of inflammatory-mediated ileus[13,21,48]. In rat studies on postoperative ileus, a role for prostaglandins was proven by the presence of COX2 mRNA and protein in residential macrophages, recruited monocytes and in a subpopulation of myenteric neurons[49]. This study also demonstrated an amelioration of the gastrointestinal motor function by treatment with the COX2 inhibitor, DFU[49]. Other animal studies, also from our own group, showed a differential effect of different COX inhibitors on postoperative motility induced by laparotomy alone or laparotomy with bowel manipulation, suggesting a possible involvement of both COX1 and COX2 isoforms and different sites of action in different stages of postoperative ileus[50,51]. A recent clinical trial comparing the effect of diclofenac (standard non-steroidal anti-inflammatory drug) and celecoxib (COX2 selective inhibitor) in patients after abdominal surgery also showed a differential effect of both drugs. Celecoxib significantly reduced the development of paralytic ileus, whereas both drugs did not result in a more rapid restoration of the gastrointestinal function compared to placebo[52]. To our knowledge, no studies of different COX inhibitors on sepsis- or endotoxin-induced ileus are available.

Moreover, prostaglandins are postulated to modulate afferent nerve signaling from the gut to the spinal cord and higher brain centers, indicating that they play a role not only in motility disturbances but also in sensitivity disturbances and pain signaling pathways. These effects are described both in the postoperative ileus model and in the endotoxin-induced ileus model[50,53].

Cell types involved in the pathogenesis of inflammatory-mediated ileus

The initial search for the location of iNOS production in the endotoxin-induced ileus model pointed to an important role for residential macrophages[24,32]. We clearly showed the presence of iNOS in residential muscular macrophages in the stomach and ileum of LPS-treated mice[24]. Besides residential muscular macrophages, the gastrointestinal tract contains a dense population of mucosal macrophages which play a crucial role in tissue homeostasis on the one hand and in the initiation, propagation and resolution of inflammation on the other hand. Mucosal macrophages are conditioned towards an anti-inflammatory role under normal circumstances and switch towards a pro-inflammatory modus during inflammation[54-56]. The relationship and the interaction between muscular and mucosal residential macrophages in the gastrointestinal tract have not been studied so far. In the field of ileus, research is focussed largely on the muscular population. Several groups have hypothesized that LPS initiates an inflammatory cascade consisting of the activation of the normally quiescent network of residential muscularis macrophages, resulting in the production of a plethora of inflammatory cytokines, chemokines and other substances such as nitric oxide and prostaglandins[13,23,24,32,33,43,48]. This inflammatory milieu results in the recruitment of circulating leukocytes and consequently in the further release of leukocyte-derived substances such as nitric oxide and prostaglandins capable of altering gastrointestinal motility[23,48] and activating inhibitory neurogenic reflex pathways[57]. The presence of iNOS is not only demonstrated in residential macrophages but also in the recruited leukocytes, thereby augmenting the inhibitory effects on gastrointestinal motility[21,58]. Monocyte-chemoattractant protein-1 (MCP-1), derived from the residential macrophages, is a key molecule in the recruitment of additional monocytes during endotoxemia, leading to an enhanced secretion of kinetically active substances that may alter gastrointestinal motility[59,60]. In a polymicrobial model of sepsis, such as the CLP model, this complex inflammatory response is induced within the intestinal muscularis with recruitment of leukocytes and mediators that inhibit intestinal muscle activity[48]. The activation of residential muscular macrophages within the gastrointestinal wall also plays a crucial role in the late inflammatory phase

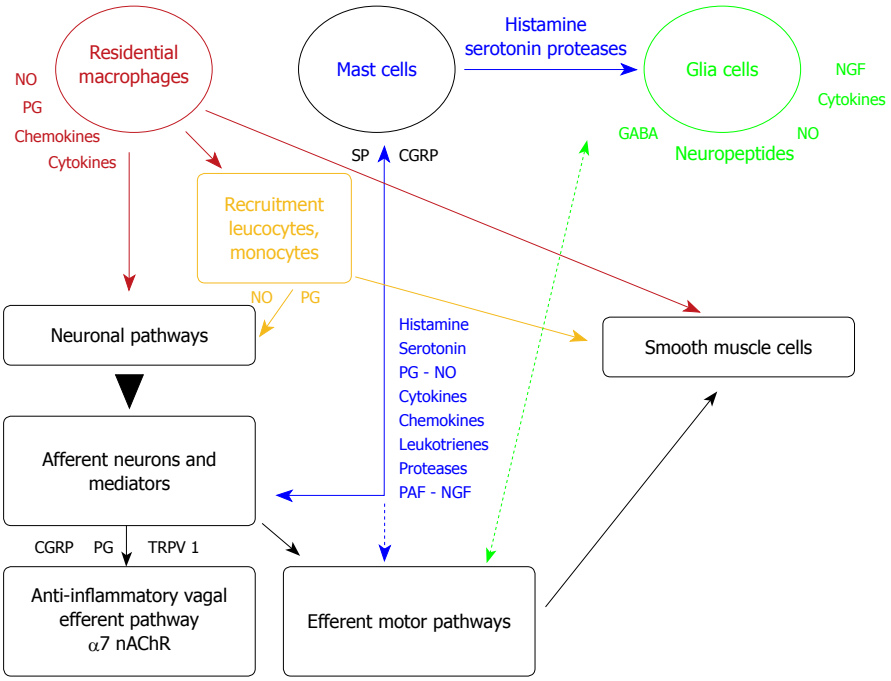

Figure 1 Hypothetical scheme of the complex interplay between, on the one hand residential macrophages (red), mast cells (blue), glial cells (green) and the recruitment of inflammatory cells (yellow), and on the other hand the activation of neuronal reflex pathways (black). Mediators involved in the different cell populations are marked in the same color as the cell type involved. NO: Nitric oxide; PG: Prostaglandins; SP: Substance P; CGRP: Calcitonin gene-related peptide; GABA: γ-aminobutyric acid; NGF: Neuropeptides, growth factors; PAF: Platelet-activating factor; TRPV: Transient receptor potential channel of the vanilloid subtype; nAChR: Nicotinic acetylcholine receptor.

of postoperative ileus, resulting in secretion of molecules such as lymphocyte function-associated antigen-1 and intercellular adhesion molecule-1 (ICAM-1), again attracting more leucocytes within the intestinal muscularis and therefore maintaining the inflammatory cascade[13,21,57]. In the postoperative model, the important role of intestinal residential macrophages was definitely proven by the work of Wehner *et al*[61], showing that depletion and inactivation of the macrophages in rats and mice prevented intestinal inflammation and postoperative ileus.

In addition to residential macrophages and inflammatory leucocytes, mast cells are also put forward as important cells in the induction and maintenance of the inflammatory cascade and its effects on motility. There is evidence for bidirectional communication between mast cells and neurons in the gastrointestinal tract[62-64]. de Jonge *et al*[65] proved elegantly that the degranulation of connective tissue mast cells is a key event in the establishment of the intestinal infiltrate in the abdominal wall in a murine model of postoperative ileus. The importance of mast cells in the pathogenesis of postoperative ileus could be translated to the human situation; The *et al*[66] showed that intestinal handling triggered mast cell activation as well as leucocyte infiltration in patients undergoing an abdominal hysterectomy. In a pilot trial with the mast cell stabiliser ketotifen, the same authors demonstrated that ketotifen improved the surgery-induced delay in gastric emptying of liquids in humans[67]. The number and activity of both residential macrophages and mast cells in LPS-treated mice is upregulated (personal communication)[68]. More studies on the role of mast cells and their mediators need to be performed in models of sepsis-induced ileus.

The question as to whether the main initiator of the inflammatory reaction is the residential macrophage or the mast cell remains unresolved. In a recent paper, Boeckxstaens *et al*[40] suggest a role for peritoneal mast cells adjacent to mesenteric blood vessels. Activated by neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) released from the adjacent afferent neurons, mast cells are able to release proinflammatory mediators into the peritoneal cavity diffusing into the blood vessels and increasing mucosal permeability. In turn, luminal bacteria and/or bacterial products enter the gastrointestinal wall and activate the resident macrophages triggering intracellular signaling pathways, leading to transcription of inflammatory molecules, cytokines, chemokines and adhesion molecules[40]. Others support a role for the residential macrophages as the first responders and conductors which would orchestrate the inflammatory events after surgical manipulation or endotoxin exposure[21]. More importantly, the interplay between these initiating cells and the nervous system should be further investigated as both the mediators released from mast cells and from residential macrophages are able to affect neuronal signaling within and from the gastrointestinal wall (Figure 1)[21,62].

Neuro-immunomodulatory pathways involved in the pathogenesis of inflammatory-mediated ileus

The gastrointestinal tract is richly innervated by an intrinsic enteric nervous system and by an extrinsic autonomic nervous system consisting of parasympathetic vagal and pelvic neurons and sympathetic splanchnic neurons. During inflammation of the gut, there is a complex multidirectional interaction between immune and inflammatory cells, neurons and smooth muscle cells (Figure 1)[21,69]. Wang *et al*[53] proved that inflammatory mediators released from the gut during endotoxemia were able to affect jejunal afferent discharge in the rat. Both LPS itself and mesenteric lymph fluid collected after injection of LPS were able to increase the afferent discharge. Liu *et al*[70] demonstrated an increased discharge in capsaicin-sensitive

mesenteric vagal afferents following systemic LPS. It was also shown that the endotoxin-induced delay in gastric emptying in rats could be suppressed by systemic capsaicin, by local application of capsaicin to the vagal nerve (but not to the celiac ganglion), and by a CGRP receptor antagonist[31]. Our own group also provided evidence for a role of afferent neurons in the motility disturbances induced by endotoxin in mice[25]. Neuronal afferent involvement was demonstrated by the beneficial effect of hexamethonium and capsaicin, and the effect of the afferent neurons was mediated by CGRP and the TRPV1 receptor. In a postoperative murine model, the involvement of both vagal and spinal afferent neurons in the inhibition of gastrointestinal motility was shown, with a differential effect of COX2 inhibition on the two types of afferent neurons[71]. All these results underline the importance of an initial activation of afferent neurons leading to the activation of inhibitory neuronal reflex pathways in gastrointestinal motility disturbances induced by sepsis or surgical manipulation of the intestine.

Activation of the vagovagal pathway is able to modulate inflammation in the gastrointestinal tract on the one hand and motility on the other hand. The cholinergic nervous system is able to attenuate the production of proinflammatory mediators and to inhibit inflammation; this mechanism is known as the cholinergic anti-inflammatory pathway[69,72,73]. The cholinergic anti-inflammatory surveillance system starts with the activation of vagal sensory afferent fibers by proinflammatory cytokines, secreted by innate immune responses stimulated *via* exogenous and endogenous molecular products of infection and injury such as LPS. Information is transmitted to higher brain centres. In the brain, vagal efferent fibers are activated; signaling back to the gastrointestinal tract. Acetylcholine inhibits the cytokine release directly *via* the α7 nicotinic acetylcholine receptor (nAChR) expressed on macrophages. Animal models showed that the anti-inflammatory effect is not exclusively mediated *via* macrophages but that other immune cells such as dendritic cells and mast cells may also be involved[73]. However, anti-inflammatory properties of vagal activation were also shown in murine isolated intestinal and peritoneal macrophages in the light of inflammatory surveillance: whereas acetylcholine stimulated the phagocytic potential of the macrophages, it inhibited the immune reactivity, as evidenced by reduction of NF-κB and proinflammatory cytokines and stimulation of IL10 production *via* nAChR α4/β2[74]. On the other hand, there is also evidence for indirect modulation of inflammatory processes *via* postganglionic neuromodulation of immune cells in primary immune organs such as the spleen[69,72,75]. In a lethal rat endotoxemia model, direct electrical stimulation of the peripheral vagus nerve was shown to inhibit tumor necrosis factor (TNF) synthesis in the liver, attenuate peak serum TNF levels and prevent the development of shock[76]. In a rat cecal ligation and puncture model, stimulation of the caudal vagal trunk prevented the induced hypotension, alleviated the hepatic damage and plasma TNFα production but had no effect on liver NF-κB activation[77]. In a murine sepsis model, transcutaneous vagal nerve stimulation reduced TNFα levels and improved survival[78]. In a murine postoperative ileus, stimulation of the vagal nerve ameliorated surgery-induced inflammation and ileus, whereas AR-R17779, an α7AChR agonist, prevented postoperative ileus and reduced the inflammatory cell recruitment in a similar mouse model[79,80].

Furthermore, sympathetic nerves might be involved in the neuroimmunomodulation of the different functions of the gastrointestinal tract. Hamano *et al*[81] showed that yohimbine, an α2-adrenergic receptor antagonist, improved endotoxin-induced inhibition of gastrointestinal motility in mice. They suggest the mechanism of action is related to the activation of α2-adrenergic receptors on macrophages downregulating the expression of iNOS. However, it could not be excluded that gastric emptying was improved *via* inhibition of the presynaptic α2-adrenergic receptors on cholinergic vagal nerves. Under normal conditions, these receptors decrease the release of acetylcholine and thereby reduce gastrointestinal motility[81]. Nevertheless, Vanneste *et al*[82] could demonstrate that presynaptic α2-adrenergic receptor control of cholinergic nerve activity was unchanged in a rat model of postoperative ileus. Also in the postoperative ileus model, a beneficial effect of spinal cord stimulation at the level of T5-T8 segments was recently shown on gastric emptying, although the mechanism of action remains to be unravelled[83]. An interaction of sympathetic neurotransmitters with the gut immune system, glial cells and gut flora was recently suggested, in correlation with the vagal immunomodulatory mechanisms in conditions of inflammation or ileus[84].

A cell population that might be relevant to consider in this neuroimmunomodulatory framework is the enteric glial cell population. Enteric glial cells are part of the enteric nervous system, along with neurons and interstitial cells of Cajal (ICC); originating from the neuroectoderm. They form a widespread network in the gastrointestinal wall where they outnumber the neuronal cell population at the level of the myenteric plexus, the submucosal plexus and the interconnecting nerve strands. Glial cells are small, star-shaped cells with numerous processes extruding from the epithelium and can be identified by the presence of specific proteins such as glial fibrillary acidic protein (GFAP), vimentin, S100B and glutamine synthetase. Glia contain precursors for neurotransmitters such as GABA and NO, express receptors for certain purinoceptors, express cytokines - interleukin (IL)-1β, IL-6, TNFα - and neuropeptides such as neurokinin A and substance P after activation[85]. Enteric glial cells can directly or indirectly modulate neuromuscular transmission, gastrointestinal motility and secretion. They also control - together with enteric neurons - intestinal barrier functions and gut immune homeostasis. Glial cells should therefore be recognised as important players in the multidirectional interactions between neurons, immune cells and intestinal epithelium (Figure 1)[85,86]. Ablation of glial cells in adult transgenic mice results in a fulminant and lethal jejunoileitis characterized by an increased myeloperoxidase activ-

ity, degeneration of myenteric neurons and intraluminal hemorrhage, pointing towards an important role of enteric glia in the maintenance of bowel integrity[87].

LPS administration in mice increases the expression of S100B in the intestine, which is indicative of an upregulation of glial cells. This effect of LPS is reversed by the cannabinoid cannabidiol, paralleled by a decrease in glial cell hyperactivation and a decrease in mast cells and macrophages (personal communication)[68]. The role of enteric glia and S100B in human gastrointestinal inflammation has been recently investigated in human biopsies, showing increased S100B in the duodenum of patients with celiac disease and in rectal biopsies of patients with ulcerative colitis; both associated with an increase in iNOS protein expression and nitrite production[88,89]. Changes in enteric glial cells and their markers (GFAP and S100β) have also been described in inflammatory bowel disease (IBD)[86].

Intracellular signaling pathways involved in the pathogenesis of inflammatory-mediated ileus

Intracellular signaling pathways play an important role in the initiation of the inflammatory immune response. Luminal bacteria and/or bacterial products enter the gastrointestinal wall and activate the resident macrophages, inducing phosphorylation of MAP kinase (ERK1/2, JNK and p38), thereby activating intracellular transcription factors such as NF-κB, STAT3, Egr-1 and NF-IL6 in both macrophages and leucocytes[21,40,79,90,91]. This leads to the induction of numerous inflammatory molecules (iNOS and COX2), cytokines (TNFα, IL1β, IL6), chemokines (MCP-1, GM-CSF, MIP1α, VEGF) and adhesion molecules (ICAM-1).

Inhibition of protein tyrosine kinases (PTK), resulting in the phosphorylation of tyrosine residues on proteins, occurs at multiple steps in the signaling cascade. Tyrphostin AG 126, a PTK inhibitor, reduces the inflammatory mediator expression induced by surgical manipulation probably through inhibition of the transcription factor NF-κB[92]. Wehner *et al*[93] showed the contribution of early p38-MAPK activation in murine postoperative ileus by the use of the macrophage specific inhibitor, semapimod. A role for Egr-1 was demonstrated in murine postoperative ileus in Egr1 knockout mice and in mice treated with the PPARγ agonist, rosiglitazone[90,91]. In addition, the beneficial effects of CO-releasing molecules in the development of postoperative ileus seem to be mediated by interference with p38 and ERK1/2 activation[94]. These data provide evidence for a key role of activation of transcription factors in postoperative ileus.

These intracellular signaling pathways also play a crucial role in endotoxic ileus. However, during sepsis and endotoxemia, TLRs come into sight and might represent an important pathogenic tool. Cells of the innate immune system recognize microorganisms and/or parts of microorganisms and initiate responses through PAMP binding to PRRs. TLRs are a family of PRRs (similar to, for instance, NOD), while PAMPs are often cell-wall molecules. LPS, a specific PAMP from Gram-negative bacteria, is known as a potent TLR4 ligand[9,95]. Specific TLR pathways (TLR2, TLR4, TLR5) are under investigation in the pathogenesis of gastrointestinal ileus in sepsis models[96-98].

Downstream intracellular signaling pathways after TLR4 activation involve different adaptor molecules (for a schematic overview see[97,99]). The bacterial molecules are presented more efficiently to the innate immune system by the complex of LPS-binding protein, CD14 and MD2, forming an essential part of the LPS-receptor next to TLR4[95,100]. Further on, stimulation of TLR recruits the adaptor molecule, myeloid differentiation primary response gene 88 (MyD88), to the receptor complex, leading to the activation of IL1R-associated protein kinases and TNF-receptor-associated factor 6 to finally activate NF-κB and MAP kinases, resulting in the production of proinflammatory cytokines and chemokines[97,99,101,102]. MyD88 plays a key role in the cytokine production in response to TLR ligands. Nevertheless, several other adaptor proteins are involved, such as TIR-domain-containing adaptor protein/MyD88 adaptor-like (TIRAP/Mal), TIR-domain-containing adaptor inducing IFNs (TRIF), TRIF-related adaptor molecule and sterile α- and armadillo-motif-containing protein[99,101,103].

Buchholz *et al*[96] recently showed the involvement of TLR4 pathways in endotoxin-induced ileus. They hypothesise that endotoxin-induced ileus is induced by TLR4 signaling in nonhematopoietic cells in the early phase (6 h after injection of LPS), whereas at high doses of LPS and at later time points both hematopoietic and nonhematopoietic TLR4 signaling contributes. The molecular response attributed to the hematopoietic cells points towards a role for residential macrophages and potentially also leucocytes in the late phase. The role of mast cells has not been investigated so far, to our knowledge. But which nonhematopoietic non-bone marrow-derived cells could then be involved in TLR4 signaling? Related to gastrointestinal disturbances, possible candidate cells are smooth muscle cells, intrinsic neurons and ICC[96]. No data are yet available, to our knowledge, regarding the expression of TLR4 on ICC. However, functional TLR4 expression is described on smooth muscle cells and myenteric plexus cells in the murine and human intestine, with expression of TLR4 on both neurons and glial cells in mice. TLR4 expression seems absent in enterocytes[104,105]. Outside the gastrointestinal wall, TLR4 receptors are also expressed in dorsal root ganglia primary sensory neurons[105] and in the rat nodose ganglion[106]. The enteric nervous system, therefore, can be directly implicated in intestinal immune defence towards intestinal microbiota.

Very recently, a dominant role for the MyD88-dependent signaling pathway in early endotoxin-induced murine ileus was shown, as MyD88 deficient mice were completely protected from endotoxin-induced ileus and the induction of the inflammatory cascade, whereas TRIF deficiency only partially protected the mice from ileus[98].

A study by Kuno *et al*[107] reported a beneficial effect on LPS-induced changes in colonic motility in the guinea pig after administration of TAK-242, a selective TLR4 signal transduction inhibitor, illustrating again the potential therapeutic options of interference with the TLR4 pathway.

POSSIBLE NOVEL THERAPEUTIC STRATEGIES

The current treatment strategies for sepsis as described in the Sepsis Guidelines 2008 were described in the first part of this paper. They largely rely on general supportive measures such as fluid resuscitation, cardiovascular support and antimicrobial treatment. The use of corticosteroids is controversial and the use of recombinant activated protein C is reserved for patients with severe sepsis and a high risk of death[2,3,10,108,109]. More specific anti-inflammatory therapy such as antilipopolysaccharide treatment and blocking of proinflammatory cytokines such as TNFα and IL1β were ineffective or have failed to improve mortality so far[108]. Selective digestive tract decontamination is not recommended in the sepsis guidelines[3]. Selective digestive tract decontamination reduces infections (mainly pneumonia) and mortality in a general population of critically ill and trauma patients, however no studies are available in patients with severe sepsis or septic shock. The juries were split on the issue of selective gut decontamination with equal numbers in favor and against the recommendation of the use of selective gut decontamination. They agreed that further research was needed in patients with severe sepsis or septic shock and they voted against inclusion in the current guidelines[3].

Therapeutic interventions related to gastrointestinal motility, secretion and epithelial barrier function might be effective as well. As ileus plays a pathogenic role in the maintenance of sepsis and multiple organ failure and in the occurrence of secondary infections, prokinetic therapy might be of value. Theoretically, ileus could be overcome, increasing gastrointestinal motility thereby reducing bacterial stasis, bacterial overgrowth and bacterial translocation and so interrupting the activation of the inflammatory cascade. Motility can, for instance, be enhanced by stimulation of excitatory neuronal pathways or by direct smooth muscle effects. For the treatment of postoperative ileus *per se*, comprehensive reviews are available in the literature[13,40,110,111]. So far, treatment of postoperative ileus is also largely supportive and based on a multimodal approach including fluid restriction, optimal (epidural) analgesia, minimally invasive surgical procedures, early mobilization and early oral feeding.

Before going into more detail regarding potential therapeutic targets, two general considerations about drug development need to be taken into account. First of all, it has been clearly shown over the last few years that the transition of promising drug targets in experimental animal models towards beneficial clinical trials is hard, difficult to predict and few products have been commercialized[112,113]. For instance, with regard to the therapeutic pipeline for irritable bowel syndrome (IBS), another disorder associated with motility and sensitivity disturbances and a possible pathogenic role for inflammation, several drugs could not finalize the research developmental trajectory towards clinical use: the neurokinin receptor antagonists and fedotozine, a peripheral κ-opioid receptor agonist, could not prove clinical efficacy in phase ⅡB and phase Ⅲ trials, although promising results were shown in experimental studies. Other drugs have been withdrawn despite clinical benefit due to safety reasons, impacting the risk-benefit ratio, such as tegaserod and alosetron. The definition of clear end points and biomarkers with clinical relevance emphasizing symptoms and quality of life need to be considered[112,113]. Secondly, interference with the patients' immune system could affect their first line defence against other infections and could affect patient wound healing[114]. In the 1990s, several controlled clinical trials of immunomodulators in severe sepsis were undertaken but failed to show benefit or even increased mortality[11,108].

As evidence for a bidirectional communication between the neuroendocrine and immune systems accumulates in the pathogenesis of inflammatory ileus (for hypothetical scheme, see Figure 1), and also in IBD and IBS, interference with the immune system seems a promising therapeutic strategy[40,64,114-117]. The interplay between the epithelial barrier function, intestinal motility and secretion, and the cellular function of immunocytes can be influenced by neuronal and immune mediators with therapeutic potential. Several potential target cells, mediators or intracellular pathways can be proposed such as mast cells, residential macrophages, glial cells, the cholinergic anti-inflammatory pathway, afferent neurons, intracellular signaling pathways such as egr-1, p38 and TLR4 transduction inhibitors, together with a plethora of neuroactive substances released by damaged or inflamed tissue such as cytokines (IL-1β, IL-6), chemokines, prostaglandins and leukotrienes, neuropeptides, growth factors (NGF), hormones, histamine, tryptase, *etc*[40,114-116]. In the field of inflammation-associated ileus, therapeutic strategies combining anti-inflammatory with prokinetic properties might have more potential for future drug development.

Ghrelin is one of these compounds with therapeutic potential, as it has been shown to possess anti-inflammatory properties together with prokinetic activity[114,118-120]. Ghrelin and the ghrelin receptor are expressed by lymphocytes, monocytes and dendritic cells. Activation of the ghrelin receptor results in an inhibition of proinflammatory cytokine expression and an increase in survival in various inflammatory disease models[114,118]. Furthermore, ghrelin and ghrelin receptor agonists are proven to be prokinetic in animal models of delayed gastric emptying and in patients with gastroparesis of different origins (for review see[119]). Experimental studies with ghrelin and clinical trials with synthetic ghrelin agonists (TZP-101, TZP-102) and a selective growth hormone secretagogue (ipamorelin) are currently ongoing. Ipamorelin, a ghrelin mimetic, proved beneficial in a postoperative ileus model in the rat[121]. In a phase ⅡB trial, the ghrelin agonist TZP-101 accelerated recovery of the gastrointestinal tract after (partial) colectomy compared to placebo[122]. Specifically related to sepsis, it was shown that ghrelin ameliorates the gut barrier dysfunction by reducing serum HMGB1 and by activation of the vagus nerve *via* central ghrelin receptors[123]. We have shown the prokinetic potential of ghrelin and the ghrelin receptor agonist, growth hormone releasing peptide 6, in a septic mouse model[26]. In a simi-

lar model, Dixit *et al*[124] showed potent anti-inflammatory effects of ghrelin on the mRNA expression of IL-1β, IL-6 and TNFα in the liver, spleen, lungs and mesenteric lymph nodes of LPS-treated mice, associated with an attenuation of the LPS-induced anorexia. The combination of prokinetic and anti-inflammatory properties enhances the potential of ghrelin-related drugs in inflammation-induced ileus. These effects are hypothesized to be mediated by the anti-inflammatory cholinergic pathway and by interactions with immune cells[118,119,120,124].

Another intestinal hormone with potential in the treatment of postoperative ileus is glucagon-like peptide 2 (GLP-2). Activation of GLP-2 was recently reported to ameliorate inflammation and intestinal dysmotility associated with surgical manipulation of the bowel in a murine model[125]. The beneficial effects on the proinflammatory milieu were more pronounced in the mucosa compared to the intestinal muscularis and the authors speculate that the protective effect of GLP-2 is associated with mucosal inflammation and barrier dysfunction, not excluding interference with the anti-inflammatory vagal pathway[125].

The endocannabinoid system is involved in the regulation of physiological and pathophysiological responses in the gastrointestinal tract such as food intake, emesis, gastric protection, gastric secretion, visceral sensation, gastrointestinal motility, intestinal inflammation and cell proliferation[126]. CB1 and CB2 receptors are the classical receptors for all kinds of cannabinoid agonists, whereas non-CB receptor-mediated effects of cannabinoids are also described[126]. Generally it is accepted that CB1 activation inhibits gastrointestinal motility in different regions of the gastrointestinal tract, whereas the role of CB2 receptors in the control of physiological motility is less clear. Both CB1 and CB2 receptors have been shown to play a role in motility in pathophysiological inflammatory conditions[126]. In septic ileus in mice and rats, CB1 and CB2 receptor antagonists protected against LPS-induced changes in motility (*in vitro* and *in vivo*), without affecting the increase in TNFα, but reduced the increase in IL6 in the group treated with a low dose of LPS[127]. We demonstrated that septic ileus in mice was associated with an upregulation of intestinal CB1 but not CB2 receptors and an increase in fatty acid amide hydrolase (FAAH), which is the principal catabolic enzyme for fatty acid amides. Cannabidiol, a non-psychotropic cannabinoid without significant binding activity to CB1 or CB2 receptors, however, further decreased the LPS-induced motility disturbances *in vivo*[128]. Very recently, we showed that LPS-induced sepsis in mice resulted in a hyperactivation of glial cells, an increase in intestinal mast cells, macrophages and TNFα. These effects were abrogated by cannabidiol treatment and associated with a decrease in S100B expression, suggesting a crucial role of glial cells (personal communication)[68].

As mast cells and residential muscular macrophages are proposed as key players in the pathogenesis of ileus, targeting these cells might also show therapeutic potential. Depletion and inactivation of macrophages in rodents prevented intestinal inflammation and postoperative ileus[61]. As interfering with immune responses could also affect wound healing as stated above, it is important to investigate the effect of macrophage depletion on the healing process. Very recently, it was shown that pharmacological and genetic inhibition of muscularis macrophages in mice did not affect intestinal anastomotic healing[129]. Whether this approach is translational to the human situation remains questionable. However, interference with the macrophages could occur at several levels. Additionally, interference with the TLR4 receptor, as described above, offers a therapeutic potential.

An interesting way to downregulate macrophages is to interfere with the cholinergic anti-inflammatory pathway; for instance, by the use of α7 nicotinic acetylcholine receptors agonists, direct vagal stimulation or the use of acetylcholine esterase inhibitors[40,69]. Electrical stimulation of the vagal nerve attenuates systemic inflammation in rodent models of endotoxemia, cecal ligation and puncture, and intestinal manipulation[76-79]. Pretreatment with the α7 nAChR, AR-R17779, prevented postoperative ileus and the inflammatory reaction in mice[80]. Nicotine itself has also been tested in clinical trials for IBD, however its use is jeopardized by its toxicity[117,130]. Electrical stimulation of the vagus nerve remains an invasive procedure and pharmacological interference with acetylcholine receptors might have side-effects; both treatment strategies need further optimization. Recently, stimulation of the vagal nerve in a rodent postoperative model by enteral administration of lipid-rich nutrition was shown to be beneficial and to be mediated by a CCK-dependent vagal mechanism[131].

With regard to mast cells, a first randomized and placebo-controlled pilot study in humans undergoing major abdominal surgery for gynecological malignancy showed that ketotifen, a mast cell stabilizer with histamine 1 receptor blocking activity, restored gastric emptying, ameliorated abdominal cramping and tended to improve colonic transit[67]. Mast cells can also be modulated at several levels including development, homing, secretory phenotype, stabilization, interference with membrane receptors or downstream pathways, or blocking the effect of the mediators released[132]. Potential drugs or drug targets are cromolyn, ketotifen, Syk kinase inhibitors; even TLR antagonists and blockers of the mediators released by mast cells such as tryptase, proteases, chymase, prostaglandins, leukotrienes, cytokines and growth factors, chemokines and neuropeptides (CRF and substance P)[63,132].

CONCLUSION

The impact of the gastrointestinal tract on the initiation and maintenance of inflammation and secondary infections involved in the pathogenesis of multiple organ failure is generally accepted. Therapeutic interventions related to gastrointestinal motility can therefore be effective in sepsis treatment, as bacterial translocation and activation of the inflammatory cascade can be put on hold. This hypothesis stresses the important link between gastrointestinal inflammation and motility.

Both endotoxic and postoperative animal models offer the opportunity to study the role of, and interference with, inflammation related to gastrointestinal motility and sensitivity. The complex interplay within the gastrointestinal wall between mast cells, residential macrophages and glial cells on the one hand, and neurons and smooth muscle cells on the other hand, forms the basis for further research towards novel therapeutic strategies. Many molecules have potential to intervene with this complex cellular interplay at the level of intracellular signaling pathways, chemokines, cytokines, neuroactive substances and mediators involved in afferent neuronal signaling and the anti-inflammatory vagal pathway. The combination of anti-inflammatory properties and prokinetic properties within one drug seems the most promising route for translational research.

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EDITORIAL

Clinical relevance of changes in bone metabolism in inflammatory bowel disease

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Abstract

Low bone mineral density is an established, frequent, but often neglected complication in patients with inflammatory bowel disease (IBD). Data regarding the diagnosis, therapy and follow-up of low bone mass in IBD has been partially extrapolated from postmenopausal osteoporosis; however, the pathophysiology of bone loss is altered in young patients with IBD. Fracture, a disabling complication, is the most important clinical outcome of low bone mass. Estimation of fracture risk in IBD is difficult. Numerous risk factors have to be considered, and these factors should be weighed properly to help in the identification of the appropriate patients for screening. In this editorial, the authors aim to highlight the most important clinical aspects of the epidemiology, prevention, diagnosis and treatment of IBD-related bone loss.

Key words: Inflammatory bowel disease; Crohn's disease; Osteoporosis; Osteopenia; Prevention

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INTRODUCTION

Low bone mineral density (BMD) is an established complication in patients with inflammatory bowel disease (IBD). Data regarding IBD-related osteoporosis needs further clarification.

Epidemiological data of bone loss are very conflicting, due to different study methodologies, patient groups, geographical distribution, *etc*[1-3].

Fracture is the most important clinical outcome of low bone mass. However, estimation of fracture risk in IBD is difficult. Numerous risk factors need to be considered, and these factors should be weighed properly to obtain relevant answers to the questions of the clinicians. If possible, fractures should be prevented, but excessive drug use should be avoided. There are also national guidelines available on the screening, diagnosis, treatment and follow-up of bone loss in IBD patients. However, some of these protocols are based on results obtained from idiopathic and postmenopausal osteoporotic patients in the general population.

In this editorial, the authors aim to summarize the available epidemiological data, identify the appropriate patients for screening for low bone mass at diagnosis and during follow-up, and review the available therapy.

EPIDEMIOLOGICAL DATA

The prevalence of low bone mass in IBD shows a wide variation in the published literature for several reasons. Of note, even diagnostic criteria for osteoporosis and low bone mass were different in the early epidemiological studies.

The gold standard for the measurement of BMD is dual X-ray absorptiometry (DEXA). BMD values are expressed in relation to the young adult mean (T-score) or age-matched controls (Z-score). Low bone mass (or osteopenia) was defined by the World Health Organisation (WHO) in 1994[4] as the value of BMD more than 1 standard deviation below the young adult mean, but less than 2 standard deviations below this value (T-score < -1 and > -2.5). WHO defines osteoporosis as a value of BMD 2.5 standard deviations or more below the young adult mean (T-score < -2.5). Using the Z-score to define osteoporosis in IBD (Z-score of < -2) seems more logical in clinical practice, because most of the patients are diagnosed with IBD before reaching the peak bone mass. However, epidemiological studies performed more recently use the WHO definitions for evaluating the rate of osteoporosis and low bone mass in IBD.

The prevalence and pathogenesis is very different in the 2 main types of IBD [Crohn's disease (CD) and ulcerative colitis (UC)], but unfortunately, most of the early studies show cumulative epidemiological data[1-3,5]. Osteoporosis and low bone mass or a Z-score less than -2.0 has been found in as many as 30.6% of 75 unselected IBD cases[1]. Similarly, the rate of osteoporosis defined by a T-score below -2.5 was 15% in a very similar IBD population[5]. Further uncontrolled studies reported the incidence of osteoporosis in CD as low as 12%[6,7], but the incidence of osteoporosis was estimated to be as high as 18%-42%[8,9] in unselected cohorts.

Other studies showed that altered bone metabolism was more frequent in CD than in UC[10,11] at diagnosis. Jahnsen *et al*[10] reported that mean Z-scores were significantly lower in patients with CD compared to patients with UC or healthy subjects. Low BMD can be a feature of CD at the time of diagnosis[11], while the impact of steroid treatment leading to low BMD seems to be more significant in UC. The main limitation of both the early and recently performed epidemiological studies is the limited number of patients.

In addition, epidemiological data depend also on the site of BMD measurement. BMD was reported to be lower at the hip than at the spine in most studies[8,9,12]. Therefore, in contrast to postmenopausal and corticosteroid-induced osteoporosis, IBD-associated osteoporosis may be at least as common at the hip. Furthermore, osteoporosis has a north-to-south gradient in the normal population. Therefore, the geographical location of the referred population in the IBD-related bone studies should also be considered as a possible confounder, e.g. low bone mass was found to be 58% in a Dutch cohort[7], while it was 32.4% in a Turkish population[13].

Fracture risk in IBD

The clinical consequence of osteoporosis is increased risk of fractures. Osteoporosis is more predictive of bone fracture than is high cholesterol level in predicting myocardial infarction.

Studies performed in the general postmenopausal osteoporotic population indicate that the risk of fracture approximately doubles for each SD reduction in BMD. The increase in fracture risk for a specific change in BMD depends on the technique used, the site measured, and the fracture type. Measurements at the hip predict hip fracture with greater power than do measurements at the lumbar spine or forearm. An increase in this site-specific relative risk (RR) is estimated to be as high as 1.5 to 3.0 for each SD decrease in the general population[14], but age is also an important predictive factor[15]. Hip fractures have long been associated with an increased mortality rate in the general population but excessive mortality has also been shown to accompany non-hip fractures and low bone mass[16].

There are only a few population-based studies regarding the fracture risk in IBD populations. In a Canadian population-based study the incidence of fracture among persons with IBD has been shown to be 40% greater than in the general population[17] [RR: 1.41, 95% confidence interval (CI): 1.27-1.56]. The RR for hip and spine fractures was 1.47 (95% CI: 1.03-2.10) and 1.54 (95% CI: 1.04-2.3), respectively in CD and 1.69 (95% CI: 1.26-2.28) and 1.9 (95% CI: 1.36-2.65), respectively in UC. In another North American study from Olmsted County, the RR for an osteoporotic fracture was as high as 1.4 (95% CI: 0.7-2.7) in CD patients compared to matched controls[18]. The risk of spine fracture was shown to be even higher (RR: 2.2, 95% CI: 0.9-5.5).

A Danish population-based study[19] evaluated the fracture risk in IBD patients compared to age- and gender-matched controls. The RR of fractures requiring hospitalization was 1.19 (95% CI: 1.06-1.33) in CD patients, and 1.08 (95% CI: 0.97-1.20) in patients with UC. The risk of spine fracture was higher compared to the risk of hip fracture (1.87 *vs* 1.1). The main limitation of this study was that fractures which did not lead to hospitalization (e.g. of the radius) were not taken into account.

All the above-mentioned studies show that IBD patients are exposed to an increased risk of fracture over matched control populations, and the greatest increased risk is found in elderly patients with IBD. A smaller Danish study[20] found that female gender, postmenopausal status, a family history of fracture and current smoking have an impact on fracture risk with RR of 2.5, 1.87, 2.4 and 1.3, respectively. The hazard ratio (HR) increased by 1.3-fold (95% CI: 1.1-1.5) in the Olmsted County study per decade.

Vertebral fractures are frequently asymptomatic; occur spontaneously or after minimal trauma, for example coughing, bending or lifting. Their incidence may be underestimated in all populations. In the general population it is estimated that only one-third of spine fractures are diagnosed. Similarly, approximately 14% of all spine fractures in CD patients were asymptomatic in a European/Israeli study[21]. The fracture rate was very similar in patients with low bone mass compared to patients with normal BMD. The fracture rate correlated with age in females but not in males.

A minority, 4 out of 63 (6.3%) vertebral fractures caused clinical symptoms in a group of 156 CD patients with reduced BMD[22] and, in contrast to the above-mentioned European/Israeli study, lumbar BMD was significantly reduced in patients with fractures compared with those without any fractures, but BMD at the femoral neck did not show any correlation with fracture risk. Approximately one third of patients with fractures were younger than 30 years in this cohort, showing that this complication may affect young patients and deserves further clinical attention.

Another important risk factor is the medical therapy used, especially corticosteroids. There is evidence that even a low dose, e.g. 2-7.5 mg prednisolone daily results in increased fracture risk[23] in any indications. A prominent role of corticosteroid therapy regarding the risk of fractures was proved in a British study. The General Practice Research Database identified all the registered IBD patients (n = 16 550) and created an age and gender matched group for evaluating the RR of fracture. The adjusted HR for hip fracture was 1.68 (95% CI: 1.01-2.78) and 1.41 (95% CI: 1.36-3.18) in CD and UC, respectively. Multivariate analysis identified both current and cumulative use of corticosteroids and the use of opioid analgesics as risk factors. This association was reported by Bernstein *et al*[24] in CD patients.

However, in a recent study of 224 CD patients, 36% had normal BMD, whereas osteopenia and osteoporosis occurred in 51% and 13%, respectively[25]. The same study demonstrated that vertebral fractures in CD patients occurred with an equal frequency in patients with low or normal BMD, regardless of corticosteroid use. Of note, the prevalence of osteoporosis in the general population is estimated to be as high as 15%. Finally, a change in the bone mass in IBD patients during short-term follow-up was shown to be low[26,27].

SCREENING

Screening for low bone mass at diagnosis is not recommended in all IBD patients. However, altered bone mineral metabolism can be observed at the diagnosis of IBD in a considerable proportion of patients, and fractures seem to correlate with BMD in the general population. Current guidelines of The American Gastroenterological Association[28] and the British Society of Gastroenterology[29] suggest determination of the risk of low bone mass and fracture individually to identify patients at risk before performing screening.

Advanced age was described to be one of the most important risk factors for IBD-associated osteoporosis, however some studies showed lower BMD at an earlier age[30,31]. Schoon *et al*[7] observed a greater risk of reduced BMD in patients with CD aged less than 18 years at diagnosis in comparison to those diagnosed at over 18 years, in their Z-score-based evaluation. Of note however, peak bone mass is achieved usually at the third decade of life.

Osteoporosis is more frequently in males with CD and UC as well. The incidence of male hypogonadism was observed to be as high as 6% (3 of 48 CD patients) in an early study[32] by measurement of serum testosterone and gonadotrophin hormone concentrations.

The duration of disease may have a valuable impact on bone metabolism in IBD for several reasons, including disease severity and drug therapy. It has been shown that cumulative steroid dose is associated with low BMD[6,26,30,33]. Corticosteroids are more often given to patients with frequent relapse of the disease, but an increased level of inflammatory cytokines seems to be an independent risk factor for accelerated bone loss[34,35]. Overall, duration, severity of disease, and corticosteroid use are difficult to separate as independent factors associated with reduced BMD.

The UK Consensus Group recommended DEXA in all patients taking 7.5 mg or more of prednisolone daily for 6 mo or more and suggested medical therapy with bisphosphonates if the T-score was < -1.5[36]. Steroid requirement is often unpredictable and bone loss associated with steroids may occur early, thus DEXA screening is recommended in all patients aged < 65 years when steroids are prescribed[29].

Of note, treatment with budesonide was associated with significantly higher BMD compared with prednisolone, in patients with active ileocecal CD[37].

Disease site (small bowel, ileum, colon) had no effect on BMD[6,11,38], and furthermore, the existence of low bone mass was observed in CD with fistulizing behavior[39].

Studies investigating the vitamin D status in patients with IBD reported conflicting results. The physiological concentration of 25(OH)-vitamin D was observed in some studies[11,40,41], while others reported reduced levels[42,43].

In summary, the guidelines suggest identifying at risk patient groups in whom DEXA screening is recommended. Screening densitometry should be performed in all IBD patients who are postmenopausal, male patients older than 50 years, in patients who receive corticosteroid therapy for more than 3 mo and in patients with a history of low trauma fracture or symptoms of hypogonadism[28]. Kornbluth *et al*[44] concluded that implementation of the guidelines led to the detection of low bone mass in a majority of patients who met the guidelines' criteria for DEXA screening.

PREVENTION AND TREATMENT

General and disease-specific risk factors may play a role in IBD-related low bone mass. Some can be modified[45], while others, such as age, genetics[46], and previous bowel resection[38] cannot be altered. Data indicate that CD-associated osteopenia can be correlated with the basic pathology of the disease itself rather than malabsorption or complications of steroid treatment[47]. Elevated local tumor necrosis factor-α (TNF-α) and other systemic inflammatory cytokine concentrations seem to be a common pathological pathway between CD- and IBD-associated osteoporosis[39,48].

Lifestyle changes can modify the BMD in IBD. Smoking cessation[49,50] and avoiding the consumption of excessive amounts of alcohol[51] are beneficial. Benefits of regular, low-impact exercise on bone mass have been proven in a randomized controlled trial[52].

Treatment should be offered if there is a reduced BMD, and other risk factors for fracture are present. However, specific treatment is licensed only for postmenopausal osteoporosis in most countries.

Adequate calcium intake has been suggested to be an important determinant of bone mass[53,54], however there was no correlation between the intake of calcium and BMD in CD patients[55,56].

Efficacy of vitamin D in the prevention of bone loss in IBD was investigated by Vogelsang *et al*[57]. There was no significant change in bone density in patients receiving 1000 IU/d vitamin D for 1 year, but significant bone loss was observed in the control group. Bernstein *et al*[58] analyzed the efficacy of this strategy in patients treated with glucocorticoids. Twenty-four patients were randomized to receive vitamin D and calcium (125 IU and 500 mg, respectively) or no treatment. There were no significant differences in BMD of the femoral neck and the vertebrae at 1 year. Calcium and vitamin D intake was not a predictor of bone status in premenopausal women; however their intake was less than the recommended dose[55]. In our study, calcium and active vitamin D supplementation was beneficial in changing markers of bone turnover (collagen crosslinks and osteocalcin) short-term in patients with active CD[59].

The active form of vitamin D is the hydroxylated 1,25(OH)$_2$-vitamin D. There are only limited data available regarding the presumable advantage of 1,25(OH)$_2$-vitamin D in CD-related osteoporosis. The efficacy of different forms of vitamin D was examined in a small cohort of CD patients in a Hungarian study[59]. The authors could show that 1,25(OH)$_2$-vitamin D had a prominent short-term beneficial effect on bone metabolism compared to 25(OH)-vitamin D.

In summary, editors of the guidelines recommend adequate calcium (1000-1500 mg/d) and vitamin D (400-800 IU/d) supplementation for IBD patients[28,29]. Efficacy of the substitution should be monitored by measuring serum calcium, 25(OH)-vitamin D and parathyroid hormone concentrations, even in CD with extensive small bowel involvement. In addition, the more frequent occurrence of kidney stones should be considered in IBD.

Earlier studies indicated that hormone replacement therapy in postmenopausal women with IBD may be favorable. Clements *et al*[60] showed a beneficial effect of hormone replacement therapy in 47 postmenopausal women with IBD, however 20 who received corticosteroids showed a smaller increase in BMD at the spine. Estrogen as monotherapy or in combination with progestin can be applied, but risk factors of myocardial infarction and stroke should be considered, as well as regular screening for breast cancer.

Bisphosphonates are the most potent anti-resorptive agents and are widely used for the treatment of osteoporosis and prevention of fractures in the general postmenopausal women population. They are also effective in the prevention of steroid-induced osteoporosis. Alendronate, risedronate and ibandronate are all proved to be effective in the therapy of IBD-associated osteoporosis[61-63]. The Royal College of Physicians guidelines[64] recommend that treatment with a bisphosphonate should be considered for all aged over 65 years on commencing steroids (or those under 65 who have already had a an osteoporotic fracture).

Administration of a TNF-α inhibitor was associated with improved BMD in CD. Long-term maintenance therapy improved BMD[65,66], and infliximab improved the bone metabolism in CD with inflammatory[35] and fistulizing behavior CD[39]. These data prove that TNF-α plays a fundamental role regarding CD-related bone loss. In the case of corticosteroid dependency, any corticosteroid-sparing agents (azathioprine, 6-mercaptopurine and methotrexate) may be of value, but a direct beneficial effect has only been proven with anti-TNF-α agents. However, bone loss itself is not an indication for anti-TNF therapy in IBD.

There is a lack of data regarding the therapeutic efficacy of calcitonin, strontium ranelate and recombinant parathyroid hormone administration in IBD patients, but there is no theoretical reason why these treatments would not be as effective as in postmenopausal women.

FOLLOW-UP

DEXA is not only the gold standard for the diagnosis of low bone mass, but it also seems to be the most appropriate method for follow-up. The most important limitation of this modality is that assessing small changes is difficult. The minimum relevant change is 3%-5% at the spine, and 4%-6% at the hip. In addition, the quality of the bone is also important and is not adequately measured by DEXA.

IBD patients who receive optimal calcium and vitamin D supplementation should undergo repeated DEXA measurements every 2 years. In the case of further bone loss despite appropriate supportive therapy, the initiation of antiresorptive therapy should be considered.

Corticosteroid-induced bone loss continues at a slower rate after the first year of therapy[67], therefore DEXA is suggested in each subsequent year of corticosteroid use or until the intervention threshold (e.g. T-score < -1.5) has been reached[29].

Serum osteocalcin, bone specific alkaline phosphatase and carboxyterminal polypeptide of type 1 collagen are used as markers of bone formation, whereas collagen degradation products such as urinary deoxypyridinoline, the carboxypeptidase of type 1 collagen and N-telopeptide cross-linked type 1 collagen are indicators of bone resorption.

Data regarding the usefulness and cost-effectiveness of these markers of bone metabolism in the diagnostic workup and the follow-up are controversial[68,69] because

of the heterogeneity in patients cohorts, methods and markers used in the different trials. Osteocalcin levels have been reported to be reduced by some authors[12,40], but normal concentrations were observed by others[9,70]. Type 1 collagen degradation products were shown to be increased[9,12] or normal[71] or elevated[70] as well. These conflicting data suggest that behavior of the disease, clinical status, medical therapy and some possible other factors may affect the bone turnover in IBD patients. It is possible that bone turnover is increased and resorption is predominant in patients with active disease, whereas low bone turnover is more prevalent in patients with quiescent disease.

None of the above-mentioned studies suggests the use of these serum or urinary markers in the diagnostic or follow-up workup in everyday practice.

The gastroenterologist should keep in mind that patients receiving any of the above-mentioned therapies should undergo appropriate screening examinations. Patients on calcium and vitamin D should be screened for kidney stones and nephrocalcinosis, but the interval of the screening is not well defined. All women receiving hormone replacement therapy should be followed up regularly for gynecological malignancies, and the risk-benefit ratio should also be considered and discussed.

CONCLUSION

Bone turnover is altered in patients with IBD. Many important risk factors (e.g. age, disease severity, medical therapy) have been identified, but a significant proportion of data regarding the diagnosis, therapy and follow-up of low bone mass in IBD have been extrapolated from postmenopausal osteoporosis. General and disease specific risk factors should be considered, particularly in patients with CD, and patients at risk of fractures should be identified and selected for diagnostic procedures. Lifestyle changes and adequate calcium and vitamin D supplementation should be introduced, and cessation of smoking is mandatory. Bisphosphonates should be considered in patients with existing osteoporosis, advanced age and long-term corticosteroid therapy. The vigilance of the physician, appropriate screening and follow-up, supportive therapy and specific control of the bone loss are all important for the optimal treatment of this complication in patients with IBD.

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EDITORIAL

Treatment strategies for nodal and gastrointestinal follicular lymphoma: Current status and future development

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Abstract

In recent years, therapies for follicular lymphoma (FL) have steadily improved. A series of phase Ⅲ trials comparing the effect of rituximab with chemotherapy *vs* chemotherapy alone in treating FL have indicated significant improvements in progression-free survival (PFS) and overall survival. Recent studies have found that prolonged response durations and PFS were obtained with maintenance therapy using rituximab or interferon after completion of first line therapy. For patients with relapsed or refractory FL, phase Ⅱ studies have assessed the effectiveness of combination therapies using a Toll-like receptor-9 agonist (1018ISS), oblimersen sodium (a Bcl-2 antisense oligonucleotide), bendamustine, and rituximab, as well as veltuzumab, a new humanized anti-CD20 antibody, and epratuzumab. In addition, the effectiveness of yttrium-90 ibritumomab tiuxetan and iodine-131 tositumomab as radioimmunotherapies has been reported. Furthermore, three phase Ⅲ studies on an idiotype vaccine are near completion. Unfortunately, these vaccines, which appeared highly effective in phase Ⅰ and Ⅱ trials, do not appear to result in prolonged PFS. This report will summarize the current knowledge on therapies for treatment of FL, and will conclude with a brief discussion of feasible future options for effective treatments. Lastly, we added descriptions of the management of gastrointestinal FL, which is considered to be controversial because it is rare.

Key words: Anti-CD20 monoclonal antibody (rituximab); Follicular lymphoma; Idiotype vaccines; Immunoradiotherapy; Treatment strategies

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INTRODUCTION

Indolent lymphoma is a lymphoma that tends to grow and spread slowly, in contrast to an aggressive lymphoma which grows and spreads quickly (Definition of Indolent lymphoma. MedTerms™: the medical reference for Medicine Net.com. http://www.medterms.com/script/main/hp.asp). While the majority of indolent lymphomas, which make up 30%-35% of all non-Hodgkin's lymphoma (NHL) subtypes, are follicular lymphoma (FL)[1], their incidence is low in Western, Asian, and less-developed countries[2]. The incidence of FL accounts for only about 6.7% of all malignant lymphomas in Japan[3]. Its incidence is rapidly increasing in Western and Asian countries[4].

FLs are often detected by the swelling of systemic lymph nodes in adults. FLs are pathologically characterized by medium-sized centrocytes and large-sized centro-

blasts found in normal follicles as proliferative cells. Most FLs are detected at an advanced stage (Ann Arbor stage Ⅲ or Ⅳ), and it was considered difficult to find a better treatment than the "watch and wait strategy" during the asymptomatic phase[5-8]. In recent years, various novel treatment options have been developed for patients with advanced stage FL, including (1) monoclonal antibodies (rituximab, veltuzumab (a new humanized anti-CD20 antibody), and epratuzumab) with conventional chemotherapy; (2) radioimmunotherapy (yttrium-90 ibritumomab tiuxetan and iodine-131 tositumomab); and (3) idiotype vaccines. These therapeutic procedures have a major impact on overall survival (OS) of patients.

Hiddemann *et al*[9] published a review article on the treatment of FL in 2005, however, in recent years, a review article especially on the recent progress in therapeutic procedures for FL has not been published. In this paper, current knowledge regarding therapies for treating FL will be summarized. By reviewing recent publications (in particular, those published in the past 2 years), a variety of treatment options, including novel monoclonal antibodies, immunoradiotherapy, combination therapies of these with conventional chemotherapies, and idiotype vaccines will be discussed. Idiotype vaccines are a nascent treatment alternative for which phase Ⅲ studies are now near completion[10]. If successful, these vaccines can potentially be developed as customized anti-cancer treatments optimized for each patient.

The gastrointestinal (GI) tract is the most common site of extra-nodal NHL, accounting for 30%-40% of primary extra-nodal NHL[11,12]. The histologic subtypes in most primary GI-NHL are mucosal-associated lymphoid tissue (MALT) lymphoma, or high-grade and aggressive B-cell lymphoma[13-16]. GI-FL is rare, and the frequency of this entity accounts for around 2% of GI-NHL[16-18]. However, recently, because capsule endoscopy and double balloon endoscopy of the small intestine were generalized widely in Japan, the number of primary GI-FL patients has tended to increase. In this review manuscript, I describe the treatment strategies for primary GI-FL, which remains controversial.

TREATMENT IS GUIDED BY THE HISTOLOGIC SUBTYPE AND EXTENT OF DISEASE

At present, the following factors are taken into account when deciding the course of treatment for FL: (1) histopathological types and grade of malignancy; (2) lesions where FL developed (in particular, those of extra-nodal origin); and (3) staging classification (Ann-Arbor classification).

Histopathological types and grade of malignancy

In FL, pathological subtypes are classified as grade 1-3a and 3b depending on the number of centroblasts per field of view. FL grade 3b, which is based on the presence or absence of residual centrocytes (3a *vs* 3b), was optional in the 2001 WHO classification[4], but is now mandatory[19]. Details of the grade of malignancy are shown below: grade 1: Number of centroblasts is 0 to 5 per high-power histological view; grade 2: Number of centroblasts is 6 to 15 per high-power histological view; grade 3: Number of centroblasts is more than 15 per high-power histological view; grade 3a: Centrocytes are present; grade 3b: Centroblasts proliferate in sheet formation and no centrocytes are present.

In nodal FL, several studies suggest that this histological grading is a good predictor of prognosis[20,21]. However, the treatment is not decided directly by this histological grading alone, and is decided mainly by staging (extent of disease) or both staging and histological grading[22].

In nodal FL, the proportions of grade 1, grade 2, and grade 3 are 40%-60%, 25%-35%, and 20%, respectively[23], while those of grade 1, grade 2 and grade 3 in GI-FL are 84.4%, 11.3%, and 4.3%, respectively[24]. The proportion of grade 1 in GI-FL accounts for about 85% and commands a majority compared with that in nodal FL. Furthermore, on staging, the proportions of stage Ⅰ and Ⅱ are 66.3% and 26.9%, respectively, and that of stage Ⅰ plus Ⅱ (early stage) is 93.2%. The degrees of grading are considered to be similar to those of staging, which is to say that in early-stage FL, the patients at stage Ⅰ and Ⅱ, and with grade 1 and 2 (Grade 1 and 2 FL is histologically subclassified as "Low-grade" FL[22]) command a majority. With regard to treatment strategies, especially in nodal FL, radiation therapy will be selected first.

In recent years, even if FL patients were found to be in the early stages (stage Ⅰ or Ⅱ), rituximab was included as a treatment strategy in those with nodal or extra-nodal FL to prolong survival, in fact, rather than the so-called "Watch and Wait strategy", aggressive therapies including mainly rituximab tend to be started in the earlier stages in Japan[25].

Lastly, in GI-FL, because the disease lesions are limited, several types of therapeutic options, for instance, surgical resections (plus adjuvant chemotherapy with rituximab, or rituximab alone), or in cases with no symptoms, chemotherapy plus rituximab or the "Watch and Wait strategy" are selected. There is no standard regimen, and the treatment policy is controversial in GI-FL[24]. Conversely, it has been reported that in nodal FL, most cases are found to be in stage Ⅲ or Ⅳ at the diagnosis with FL[22], however, the proportions of grade 1 and 2 are about 50% and 30%, respectively (the proportion of grade 1 plus 2 is 80%)[23], and the degree of grading is considered to be dissimilar to that of staging. The number of patients with stage Ⅲ or Ⅳ and low-risk or low-grade (grade 1 or 2) FL seems to be comparatively high. There is no standard therapy for advanced, but low-grade FL to date[24], however, a combination of classical chemotherapy and rituximab is now considered to be a main therapy for advanced FL, because it has been reported that this combination prolonged survival compared with several classical chemotherapies alone.

The treatments for nodal FL and GI-FL are summarized as follows: Most cases with GI-FL have been found to have focal disease and an early-stage condition at diagnosis, with a histological grading of low-grade, while nodal FL is almost always found at an advanced stage. However, the degrees of cellular malignancies were considered to be divided into two groups of low-grade and high-grade, and the proportions were reported to be about 80% and 20%, respectively. When physicians discuss the treatment strategy for nodal FL and GI-FL, they should consider the differences in the status between these two groups, however, both the treatment regimens for stage Ⅲ-Ⅳ low-grade FL, and stage Ⅲ-Ⅳ high-grade FL do not differ at present.

Furthermore, there is little or no difference in the natural clinical course or the response to treatment between the two groups with histological grade 1 and 2 FL. There is a consensus that physicians initiating different therapeutic options for FL seems meaningless[22].

Biological malignancy is also an important factor which is directly associated with the degree of differentiation of tumor cells, the increased speed of tumor cells themselves, infiltrative growth into the surrounding tissue, and liability to metastasize, which is important for prognosis. With this in mind, the following subjects should be discussed: (1) How many degrees, and how to add the factors with differences in histological grading of the tumor cells themselves, to the factors regarding the selection of therapeutic procedures induced by the differences in staging; and (2) Is the present method of grading FL cells really suitable for FL? This is one of the problems which is very difficult to solve, but very important when discussing the treatment strategies for FL in the future.

Grade 3b FL shows the same clinical features as diffuse, large B-cell lymphoma (malignant lymphoma with a medium grade of malignancy). This grade does not show a slow and indolent course, but rather the same clinical course as an aggressive lymphoma. Some studies have identified biological differences between these 2 subtypes, with most instances of FL Grade 3b being more closely related to diffuse large B-cell lymphoma at the molecular level[26,27]. Consequently, the treatment policy for Grade 3b should be guided in a similar fashion to that of aggressive lymphoma. Given that tumor progression is very different, Grade 3b FL needs to be distinguished from the other grades of FL.

Clinical staging of FL

Clinical staging of nodal FLs is determined using the Ann-Arbor Clinical Staging system[28], while GI-FLs are classified by the Lugano staging classification of GI tract lymphoma[29], which is the modified version of the Ann-Arbor staging system for GI tract lymphomas.

Most indolent lymphomas like FL are discovered at an advanced stage because they grow slowly and only a small number of FL cases have systemic symptoms.

Most FLs are detected at an advanced stage (Ann Arbor stage Ⅲ or Ⅳ), and it was considered difficult to find a better treatment than the "Watch and Wait strategy" during the asymptomatic phase[5-7].

Table 1 Lugano staging classification of gastrointestinal tract lymphoma[29]

Stage	
Ⅰ	Tumor confined to GI tract Stage primary site or multiple, noncontiguous lesions
Ⅱ	Tumor extending into abdomen from primary GI site Nodal involvement
Ⅱ$_1$	Local (paragastric in cases of gastric lymphoma and paraintestinal for intestinal lymphoma)
Ⅱ$_2$	Distant (mesenteric in the case of an intestinal primary; otherwise paraaortic, paracaval, pelvic, inguinal)
ⅡE	Penetration of serosa to involve adjacent organs or tissues [enumerate actual site of involvement, e.g. ⅡE (pancreas), ⅡE (large intestine), ⅡE (post intestinal wall)] Where there is both nodal involvement and penetration involving adjacent organs, the stage is denoted using both a subscript (1 or 2) and E, e.g. Ⅱ$_1$E (pancreas)
Ⅳ	Disseminated extra-nodal involvement, or a GI tract lesion with supradiaphragmatic nodal involvement

GI: Gastrointestinal.

However, randomized controlled trials (RCT) using a new anti-CD20 antibody have shown that combination therapy with traditional chemotherapy plus rituximab prolonged OS in patients compared with conventional chemotherapy alone. Rather than "Watch and Wait", combination therapy with monoclonal antibody and chemotherapy is considered standard therapy even when non-symptomatic FL is discovered. Even if FL patients are found to be in the early stages (stage Ⅰ or Ⅱ), rituximab has been introduced as a treatment strategy in patients with nodal FL to prolong survival, in fact, aggressive therapies including mainly rituximab have recently been initiated in the earlier stages in Japan.

Radiation therapy can treat cases in the limited stage (stage Ⅰ A). Cases need to be assessed to determine whether they are in stage Ⅰ using a PET-CT scan (difficult to detect FL), systemic CT scan, bone marrow puncture, or aspiration.

For FL cases in the limited stage (Ann-Arbor staging Ⅰ A), additional radiation therapy directed at focal lesions after 3 or 4 courses of chemotherapy can be considered in addition to the integral therapy.

In cases where a portion of the tumor remains, additional radiation therapy can be performed after chemotherapy.

Chemotherapy should be performed for cases at a stage ≥ Ⅱ.

The Lugano staging classification of GI tract lymphoma[29] is shown in Table 1.

This is the modified version of the Ann Arbor staging system for GI tract lymphomas. Yamamoto *et al*[24] reported that 128 (66.3%) and 52 (26.9%) of 193 GI-FL cases were at stage Ⅰ and Ⅱ, respectively. Surprisingly, over 90% of all GI-FL cases which have been reported in the English literature were at the early stages, and had localized disease. Yamamoto *et al*[24] suggested that this was because of the low tendency of GI-FL to disseminate outside the GI tract, and the higher frequency of grade 1 lymphoma

in GI-FL cases. Furthermore, the GI-FL cases, in which sufficient examinations and successful treatments were performed due to non-advanced stages and no symptoms, were likely to be reported and submitted for publication in the English literature. The possibility of bias in the selection of the reported cases or case series should be raised. Nodal involvement can be found in GI-FL cases, and this status is classified as stage Ⅱ1 (involvement of local lymph nodes) or Ⅱ2 (involvement of distant lymph nodes).

Disease distribution (unifocal or multifocal) is important for selecting treatments in GI-FL patients.

In recent years, Yamamoto *et al*[24], Kodama *et al*[30] and Higuchi *et al*[31] have claimed that other disease lesions in the small intestine were newly and secondarily found in over 70% of GI-FL patients who had disease lesions in the GI tract except the small intestine in which capsule endoscopy and double-balloon endoscopy were performed. If the patients were found to be multifocal, rituximab with or without conventional chemotherapy may be administered.

THERAPIES FOR NODAL FOLLICULAR LYMPHOMAS

Traditional therapies for FL

FL cases are categorized into pathological grades (1 to 3) based on the proportion of cells comprised by centroblasts. This grading system is an important factor in determining prognosis. For example, grade 3 lymphoma is the most malignant and has the fastest progression, however, chemotherapy can be an effective treatment. In contrast, grade 1 represents the least malignant lymphoma and has a very slow progression where the patient may die 10 years after the time of diagnosis. However, this lymphoma is unaffected by chemotherapy. Thus, prognosis between grades 1 and 3 is strikingly different[32-35].

In cases of NHL not restricted to FL, and in those where GI invasion is observed, patients risk lethal complications if the chemotherapy is too strong. These complications include bleeding or GI perforations due to tumor necrosis. Thus, GI complications must be considered when selecting the appropriate treatment regimen. However, choosing mild chemotherapy or low-intensity radiation therapy due to GI lesions may also result in insufficient treatment of systemic lymphoma. Therefore, the success or failure of a treatment depends on both the grade and degree of disease spread.

Several therapies have been tested for treating GI-FL. In grade 1 and 2 FLs, some treatment options include an alkylating agent alone, a combination therapy with multiple drugs, and high-dose chemotherapy in combination with hematopoietic stem cell transplantation. However, no more than 10% of patients maintain complete remission during the 5-year post-treatment period. Additionally, while the median survival time is seven to 10 years, the survival rate declines progressively and becomes lower than that of aggressive lymphoma after approximately 15 years[36]. Given that the effects of chemotherapy have so far been suboptimal, and disease progression is very gradual even without treatment, some have suggested initiating treatment only after symptoms actually emerge. In the interim, they recommend careful observation. In the early stages (Ⅰ and Ⅱ) of FL, radiation therapy has been effective, resulting in a control rate of 95% and a 10-year progression-free survival (PFS) rate of 40%-52%. In these circumstances, radiation therapy is considered standard treatment because there is promise of healing or remission for an extensive time period. However, the likelihood of radiation therapy actually being performed clinically is low, even in the US[37]. While studies are being conducted to observe the effects of rituximab and irradiation combination therapy, no long-term observational data exist. Recently, therapeutic drug-targeting molecules such as rituximab have played an important role in prolonging the lives of FL patients. In the treatment of systemic FL, 6 cycles of CHOP and rituximab (R-CHOP) combination therapy appear to be the most promising. During a phase Ⅲ RCT, R-CHOP was found to significantly prolong survival compared to CHOP therapy alone[38,39].

Randomized control studies of rituximab plus conventional chemotherapy vs conventional chemotherapy alone

Results of RCTs comparing response rate, duration, disease-free survival (DFS), and OS between patients treated with conventional chemotherapy and those treated with rituximab plus conventional chemotherapy have been reported (Tables 2 and 3). Details are described below.

Rituximab plus chemotherapy trials for previously untreated FL: Several studies have shown that conventional chemotherapy plus rituximab for previously untreated FL prolonged various types of survival (Table 2).

A multicenter randomized phase Ⅲ trial of 321 patients with previously untreated FL compared cyclophosphamide, vincristine, prednisolone (CVP) alone to rituximab plus CVP (R-CVP, $n = 162$)[40]. OS was higher with R-CVP compared to CVP alone (OS, $P = 0.029$; 4-year OS: 83% *vs* 77%, respectively).

The randomized trial conducted by the German Low-grade Lymphoma Study Group involved treating patients with previously untreated FL with 6-8 cycles of CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) combined with rituximab (R-CHOP, $n = 223$) or CHOP alone ($n = 205$). A significantly higher overall response (OR) rate (96% *vs* 90%, respectively, $P = 0.011$) and prolonged duration of remission ($P = 0.001$) were achieved with R-CHOP. The first 3-year OS with 6 deaths in the R-CHOP group was superior to that of 17 deaths in the CHOP group ($P = 0.016$)[38,39].

The FL-2000 randomized trial involved subjecting patients with previously untreated FL to treatment with either CHVP (cyclophosphamide, adriamycin, etoposide and prednisolone) plus interferon-α2a (CHVP+I arm)

Table 2 Results of different randomized trials comparing chemotherapy with chemotherapy plus rituximab in previously untreated patients with follicular lymphoma

Study	No. of patients, pathological types, staging	Median age (yr)	Complete response rate	End point				
				Median progression-free survival	Event-free survival	Median response duration	Time to treatment failure	Overall survival
CVP[40]	n = 321, FL, Stage Ⅱ-Ⅳ	52	$P < 0.0001^b$			$P < 0.0001^b$	$P < 0.001^b$	$P = 0.029^a$
R-CVP	n = 162	Unknown	41%	34 mo		Prolonged than CVP alone (lower relapse rate than CVP alone)	Prolonged than CVP alone	83% (at 4 yr)
CVP	n = 159	Unknown	11%	15 mo				77% (at 4 yr)
GLSG[38,39]	n = 428, FL, Stage Ⅲ or Ⅳ (advanced stage)	55	$P > 0.05$ (not significant)			$P = 0.001^b$	$P < 0.001^b$	$P = 0.016^a$
R-CHOP	n = 223	54	20%	Not reached		Lower relapse rate than CHOP alone	28/233 (12.0%)	95% (at 2 yr)
CHOP	n = 205	57	17%	31 mo			61/205 (29.8%)	90% (at 2 yr)
FL2000[41]	n = 358, FL, 89% > stage Ⅱ	61	$P = 0.001^b$		$P = 0.001^b$	$P = 0.012^a$		$P > 0.05$ (not significant)
R-CHVP/IFN	n = 175	Unknown	67% (at 18 mo)	Not reached	53% (at 5 yr)	64% (at 4 yr)		84% (at 5 yr)
CHVP/IFN	n = 183	Unknown	50% (at 18 mo)	35 mo	37% (at 5 yr)	44% (at 4 yr)		79% (at 5 yr)
OSHO[42]	n = 201, FL, Stage Ⅱ and Ⅳ	59	$P = 0.0004^b$	$P < 0.0001^b$	$P < 0.0001^b$	$P < 0.0001^b$		$P = 0.0096^b$
R-MCP	n = 105	60	52/105 (50%)	Not reached	Not reached	Not reached		87% (at 4 yr)
MCP	n = 96	57	25/96 (25%)	28.8 mo	26 mo	35 mo		74% (at 4 yr)

$^aP < 0.05$, $^bP < 0.01$, statistically significant. (R-)CVP: (Rituximab plus) cyclophosphamide, vincristine, and prednisone; FL: Follicular lymphoma; GLSG: German Low-Grade Lymphoma Study Group; (R-)CHOP: (Rituximab plus) cyclophosphamide, doxorubicin, vincristine, and prednisone; (R-)CHVP/IFN: (Rituximab plus) cyclophosphamide, adriamycin, etoposide, prednisolone plus interferon-α2a; (R-)MCP: (Rituximab plus) mitoxantrone, chlorambucil, and prednisolone; OSHO: East German Study Group Hematology and Oncology.

Table 3 Results of different randomized trials comparing chemotherapy with chemotherapy plus rituximab in relapsed or refractory follicular lymphomas

Study	No. of patients, target disease	Median age (yr)	Complete response rate	End point	
				Median progression-free survival	Overall survival
GLSG[43]	n = 65, relapsed FL		$P = 0.011^a$	$P = 0.0139^a$	$P = 0.0943$ (not significant)
R-FCM	n = 35	60	94%	Near 36 mo	90% (at 2 yr)
FCM	n = 30	59.5	70%	21 mo	70% (at 2 yr)
EORTC, HOVON, NCIC, CTG, BNLI, ALLG, NLG and EORTC Data Center-multicenter study[44]	n = 465, relapsed/resistant FL		$P < 0.001^b$	$P < 0.001^b$	$P = 0.096$ (not significant)
R-CHOP	n = 234	54	29.5%	33.1 mo	82.5% (at 3 yr)
CHOP	n = 231	55	15.6%	20.2 mo	71.9% (at 3 yr)

$^aP < 0.05$, $^bP < 0.01$, statistically significant. GLSG: German Low-Grade Lymphoma Study Group; FL: Follicular lymphoma; EORTC: European Organization for Research and Treatment of Cancer; HOVON: Hemato-Oncologie voor Volwassenen Nederland; NCIC: National Cancer Institute of Canada; CTG: Clinical Trial Group (Canada); BNLI: British National Lymphoma Investigation; ALLG: Australasian Leukaemia and Lymphoma Group; NLG: Nordic Lymphoma Group; (R-)CHOP: (Rituximab plus) cyclophosphamide, doxorubicin, vincristine, and prednisone; (R-)FCM: (Rituximab plus) fludarabine, cyclophosphamide, mitoxantrone.

or CHVP combined with rituximab plus interferon (R-CHVP+I arm). After a median follow-up of 5 years, event-free survival was significantly different between the two arms (37% in the CHVP+I arm *vs* 53% in the R-CHVP+I arm, $P = 0.001$). However, the 5-year OS rate between the two arms of CHVP+I and R-CHVP+I was not significantly different[41].

Herold *et al*[42] randomly assigned previously untreated patients with advanced MALT lymphoma, FL, or mantle cell lymphoma (MCL) to one of two groups, either mitoxantrone, chlorambucil, prednisolone plus rituximab (R-MCP, n = 181) or MCP alone (n = 177). All patients who achieved a complete or partial remission were treated with maintenance interferon until they relapsed. They reported the results from the primary analysis population of patients with FL alone, who constituted the majority of patients (56%) recruited to the trial (n = 201; R-MCP, n = 105; MCP, n = 96). OS rate in the R-MCP group significantly improved compared to the MCP alone group (4-year OS rate, 87% *vs* 74%, respectively, $P = 0.0096$).

Table 4 Results of different randomized trials comparing no maintenance with maintenance therapy with rituximab or interferon after first line therapy in patients with follicular lymphoma

Author(s) or studies, maintenance therapy	No. of patients, target disease and randomization to therapies	Median age (yr)	Initial therapy, *n* (%)	CR rate	End point		
					Median PFS	Median response duration	Overall survival
GLSG[43,46]	*n* = 105, relapsed or refractory FL					*P* = 0.035[a]	
R-FCM	*n* = 52	59	R-FCM: 41 (79) FCM: 11 (21)		Not reached	32/41 (78%)	
Observation	*n* = 53	61	R-FCM: 40 (75) FCM: 13 (25)		Not reached	21/40 (53%)	
ECOG1496 study[47]	*n* = 311, stage Ⅲ-Ⅳ indolent lymphoma (282 of 311 had FL)	61	CVP	*P* = 0.00006[b]		*P* = 0.012[a]	All: *P* = 0.05 FL: *P* = 0.08 (trend towards significance)
R-CVP	*n* = 153		CVP	22%		All: 68% (at 3 yr) FL: 64% (at 3 yr)	All: 92% (at 3 yr) FL: 91% (at 3 yr)
Observation	*n* = 158		CVP	7%		All: 33% (at 3 yr) FL: 33% (at 3 yr)	All: 86% (at 3 yr) FL: 86% (at 3 yr)

[a]*P* < 0.05, [b]*P* < 0.01, statistically significant. CR: Complete response; PFS: Progression-free survival; FL: Follicular lymphoma; GLSG: German Low-Grade Lymphoma Study Group; (R-)FCM: (Rituximab plus) fludarabine, cyclophosphamide, mitoxantrone; ECOG: Eastern Cooperative Oncology Group; (R-)CVP: (Rituximab plus) cyclophosphamide, vincristine, and prednisone.

Randomized trials of rituximab with chemotherapy in relapsed or refractory FL: A randomized trial which involved fludarabine, cyclophosphamide, mitoxantrone (FCM) alone or rituximab plus FCM (R-FCM)[43] was conducted with 30 FL patients allocated to the FCM and 35 FL patients to the R-FCM treatment groups (total 65 patients). The OR rate [complete response (CR) plus partial response rate] for R-FCM was 94% compared to 70% for FCM alone. The R-FCM arm was significantly superior with respect to PFS ($P = 0.0139$) compared to FCM for FL[43] (Table 3).

In another randomized trial, a total of 465 patients with relapsed or resistant FL were randomly assigned to CHOP or R-CHOP induction therapy groups. Those in complete remission or partial remission were then randomly assigned to maintenance therapy with rituximab or an observation alone group. Median PFS for rituximab maintenance or observation alone after the second randomization was significantly different (51.5 mo *vs* 14.9 mo, respectively). In relapsed or resistant FL, rituximab maintenance improves PFS after R-CHOP induction[44] (Table 3).

Maintenance therapy with rituximab or interferon after first-line therapy

The question as to whether or not maintenance therapy should be carried out has been raised. In one case involving rituximab, Hainsworth *et al*[45] concluded that maintenance therapy is not required because first-line therapy with rituximab was effective in cases without maintenance therapy. Conversely, several studies have shown that rituximab as a maintenance therapy after conventional chemotherapy or interferon as maintenance therapy after conventional chemotherapy plus rituximab prolonged response duration and PFS (Table 4).

Forstpointner *et al*[43,46] reported that maintenance therapy with rituximab was effective after salvage therapy with rituximab along with conventional chemotherapy, and significantly prolonged the response duration in patients with FL. Hochster *et al*[47] also reported the results of the randomized phase Ⅲ ECOG1496 trial, and found that maintenance rituximab after cyclophosphamide, vincristine, and prednisone prolonged PFS in patients with stage Ⅲ-Ⅳ (advanced) untreated indolent lymphoma (282 out of a total of 311 patients had FL). Among patients with FL alone, OS at 3 years in the patients with and without maintenance rituximab was 91% and 86%, respectively; HR = 0.6, log-rank one-sided $P = 0.08$). Further, Herold *et al*[42] reported that rituximab added to first-line MCP, followed by interferon maintenance, prolonged survival in patients with advanced FL.

New anti-cancer agents with rituximab in patients with relapsed or refractory FL

Several studies have recently reported on phase Ⅱ trials involving combination therapy with new anticancer agents plus rituximab for relapsed or refractory FL patients. Friedberg *et al*[48] conducted a Phase Ⅱ trial of a new anticancer agent, Toll-like receptor-9 (TLR-9) agonist, combined with rituximab in patients with relapsed and refractory FL. TLR-9 agonists have pleiotropic effects on both innate and adaptive immune systems, including increased antigen expression, enhanced antibody-dependent cell-mediated cytotoxicity, and T helper cell type 1 shift in the immune response. They reported that clinical responses were observed in 48% of patients and that the overall median PFS was 9 mo. Pro *et al*[49] conducted a Phase Ⅱ multicenter study of combination therapy with oblimersen sodium, a Bcl-2 antisense oligonucleotide, and rituximab in recurrent B-cell NHL patients. They reported an OR rate in relapsed FL patients of 60% (eight complete remissions and four partial remissions out of 20 patients), and that the combination of oblimersen sodium and

rituximab seemed to be most effective in patients with indolent NHL including FL. Further, Robinson *et al*[50] evaluated the phase Ⅱ multicenter study of bendamustine plus rituximab in patients with relapsed indolent B-cell and MCL who showed no resistance to prior rituximab. They reported that OR rate, median duration of response, and median PFS time were 92% (41% CR), 21 and 23 mo, respectively, and concluded that the combination of bendamustine plus rituximab is suitable for relapsed indolent lymphoma centering on FL and MCL.

New anti-CD20 antibody for indolent B-cell lymphoma

Veltuzumab is a humanized anti-CD20 antibody with structure-function differences from chimeric rituximab. Morschhauser *et al*[51] evaluated a multicenter phase Ⅰ / Ⅱ dose-finding trial in relapsed/refractory B-cell NHL evaluating the efficacy of veltuzumab. They reported that in FL, 24 (44%) of 55 patients had objective responses, and CR were seen in 15 (27%) patients. In marginal zone lymphoma, 5 (83%) of 6 patients had OR. Veltuzumab appeared to be safe and active at all doses tested with rituximab, including dose levels less than those typically used, encouraging further study.

New antibodies against targets other than CD20

Epratuzumab, a humanized IgG1 unconjugated anti-CD22 antibody: Leonard *et al*[52] conducted an international multicenter, phase 2 trial in patients with recurrent FL and reported durable CR with a combination of epratuzumab and rituximab. They reported an OR rate of 85% in patients with FL who had Follicular Lymphoma International Prognostic Index (FLIPI) risk scores of 0 or 1 (n = 13), whereas 28 patients with intermediate or high-risk FLIPI scores ($\geqslant$ 2) had an OR rate of 39%.

Inotuzumab ozogamicin (CMC-544), the calicheamicin-conjugated anti-CD22 monoclonal antibody and rituximab: Takeshita *et al*[53] studied the effect of CMC-544, a calicheamicin-conjugated anti-CD22 monoclonal antibody, used alone or in combination with rituximab, and investigated the quantitative alternations on the target molecules of CD20, CD22, CD55and CD59 in cells obtained from patients with B-cell malignancies or Dauji and Raji cells. The reduction in CD55 and the preservation of CD20 after incubation with CMC-544 support the rationale for the combined use of CMC-544 and rituximab[48]. This new antibody may also be used for the treatment of FL in the future.

Galiximab, a human-primate chimeric anti-CD80 antibody: Galiximab is a human-primate chimeric anti-CD80 antibody with excellent tolerability and single-agent effectiveness for recurrent FL[54]. A phase Ⅰ / Ⅱ trial of the combination of rituximab plus galiximab demonstrated a 64% response rate and a 12.1 mo median PFS[55]. A phase Ⅲ trial comparing rituximab with the combination of rituximab plus galiximab in patients with relapsed FL is ongoing.

Other potential new antibodies against targets other than CD20, CD22, or CD80: Phase Ⅰ / Ⅱ trials testing new CD52 (alemtuzumab), CD2 [MEDI-507 (siplizumab)], CD30 [SGN-30 and MDX-060 (iratumumab)], and CD40 (SGN-40)[56] antibodies are now ongoing or have been completed in aggressive lymphoma patients. It is possible that these new antibodies will be used for the treatment of FL in the near future.

Radioimmunotherapy for FL

In 2005, Kanzius *et al*[57,58] provided new systems and methods for radio-frequency-induced hyperthermia plus radioimmunotherapy using ^{90}Yttrium-ibritumomab tiuxetan or ^{131}Iodine-tositumomab for patients with NHL containing FL.

Recently, radioimmunotherapy using ^{90}Yttrium-ibritumomab tiuxetan or ^{131}Iodine-tositumomab, which are produced from anti-CD20 monoclonal antibodies combined with radioactive materials, has been performed for refractory or relapsed FL patients. Recent reports in this field are listed below:

^{131}Iodine-rituximab for relapsed indolent B-cell lymphoma (FL and MALT lymphoma): Illidge *et al*[59] evaluated a novel protocol using 4 weekly infusions of 375 mg/m^2 rituximab followed by 2 fractions of ^{131}Iodine-rituximab, preceded by a 100 mg/m^2 predose of rituximab in patients with relapsed indolent B-cell lymphoma (FL and MALT lymphoma). OR rate was 94%, with a CR rate of 50%. Median time to progression was 20 mo, which is significantly longer than that for the last qualifying chemotherapy (P = 0.001).

^{90}Yttrium-ibritumomab tiuxetan for previously untreated FL: Jacobs *et al*[60] reported on a trial involving patients with previously untreated FL who received CHOP-R (three cycles of CHOP and four additional weekly rituximab cycles). CR rate after CHOP-R, as assessed by CT and PET imaging, was 40% and 46%, respectively. CR rate further improved following ^{90}Yttrium-ibritumomab tiuxetan) to 82% and 89%, respectively.

^{90}Yttrium-ibritumomab tiuxetan for advanced FL after first remission: Morschhauser *et al*[61] described that consolidation of first remission with ^{90}Yttrium-ibritumomab tiuxetan in advanced-stage FL is highly effective with no unexpected toxicities, prolonging PFS by 2 years and resulting in high partial remission-to-complete remission conversion rates regardless of the type of first-line induction treatment.

^{90}Yttrium-ibritumomab tiuxetan for relapsed or refractory indolent B-cell lymphoma (containing FL): Tobinai *et al*[62] reported that radioimmunotherapy with ^{90}Yttrium-ibritumomab tiuxetan is safe and highly effective in patients with relapsed or refractory indolent B-cell lymphoma (small lymphocytic, lymphoplasmacytic, mantle cell, follicular, MALT, and splenic marginal zone lympho-

Table 5 Results of phase Ⅲ clinical trials of idiotype vaccines in follicular lymphoma

Study	No. of patients, target disease	Regimen of induction therapy	Eligibility	Vaccine type	Vaccine production methodology	Primary end point
						Survival types, *P*-value, significance
Biovest	*n* = 177, untreated FL	PACE or R-CHOP	CR	Id-KLH/GM-CSF	Rescue hybridoma	Disease-free survival, $P > 0.05$, not significant
Genitope	*n* = 287, untreated FL	CVP	CR, PR	Id-KLH/GM-CSF	Recombinant DNA	Progression-free survival, $P > 0.05$, not significant
Favrille	*n* = 349, untreated or recurrent FL	Rituximab	CR, PR, SD	Id-KLH/GM-CSF	Recombinant DNA	Time to progression, $P > 0.05$, not significant

FL: Follicular lymphoma; PACE: Prednisolone, doxorubicin, cyclophosphamide and etoposide; R-CHOP: Rituximab plus cyclophosphamide, doxorubicin, vincristine, prednisolone; CVP: Cyclophosphamide, vincristine, prednisolone; CR: Complete response; PR: Partial response; SD: Stable disease; Id-KLH: Idiotype-keyhole limpet hemocyanin; GM-CSF: Granulocyte-macrophage colony stimulating factor.

mas) pretreated with rituximab-based therapy. They also reported OR and CR rates of 83% and 68%, respectively, and a median PFS of 9.6 mo.

Idiotype vaccine

Idiotype vaccines are based on active immunization of patients against their own tumor idiotype, and could lead to an even more effective and sustained anti-tumor response than the passive infusion of anti-idiotype antibodies[10]. Vaccine therapies, which utilize antibodies against the characteristic tumor idiotype for each FL patient, are expected to result in anti-tumor effects, improved response rates, and prolonged survival times. These benefits are anticipated because these vaccine therapies can potentially be developed as personalized anti-cancer treatments optimized for each patient. Several phase Ⅰ / Ⅱ clinical trials for idiotype vaccines in FL have been conducted in the last 20 years, of which about 10 have been published and demonstrate the effectiveness of idiotype vaccines[63-71]. In particular, Inogès *et al*[69] reported that most vaccinated patients with FL had significantly longer DFS after second remission. These encouraging results from phase Ⅰ / Ⅱ trials initiated three phase Ⅲ RCTs (Biovest, Genitope, and Favrille). These phase Ⅲ trials are now essentially completed and have been evaluated (Table 5). While there initially seemed to be a significant prolongation of PFS during the early stages of the trials, all of these phase Ⅲ trials ultimately failed to prolong PFS[10].

McCormick *et al*[72] recently produced patient specific recombinant idiotype vaccines in plants against follicular B cell lymphoma and conducted a phase Ⅰ trial. They reported that more than 70% of patients developed cellular or humoral immune responses, and that 47% of patients developed antigen-specific responses[72]. Results from future phase Ⅱ / Ⅲ trials will be of great interest.

THERAPIES FOR GASTROINTESTINAL FOLLICULAR LYMPHOMAS

Very recently, Yamamoto *et al*[24] performed a literature review of Japanese cases with GI-FL. This review on GI-FL is well written and I believe may help with regard to the recent confusion surrounding the selection of treatment for GI-FL patients.

I would like to summarize this editorial, especially the treatment of GI-FL patients, by focusing on the main points covered in this review article.

Introduction of GI-FL

The GI tract is the most common site of extra-nodal NHL, accounting for 30%-40% of all primary extra-nodal NHL[11,12]. While GI involvement in aggressive NHL (nodal aggressive NHL with involvement of the GI tract) is common, primary GI-NHL is rare, and GI-FL is very rare. It has been reported that the histologic subtypes in most primary GI-NHL are MALT lymphoma or high-grade B-cell lymphoma[13-16]. GI-FL is rare, and this histology represents around 2% of all GI-NHL[16-18]. In addition, over 90% of GI-FL cases which have been published in the English literature to date were early stages (stage Ⅰ and Ⅱ)[24]. Very recently, Yamamoto *et al*[24] performed a review of the literature on patients with GI-FL, and claimed that the diagnosis, prognosis, and treatment of GI-FL remain largely unknown mainly because of its rare frequency. However, recently, because capsule endoscopy and double balloon endoscopy of the small intestine were generalized in Japan, the number of primary GI-FL patients has tended to increase.

Prognosis in patients with GI-FL

The long-term clinical outcome of GI-FL is as yet unclear. Damaj *et al*[73] reported that there was no significant difference between two groups of patients treated by either conventional chemotherapy with rituximab, and patients without therapy based on the "Watch and Wait" concept regarding the median time to disease progression (37.5 mo) and the median relapse-free time (31 mo).

Treatment for GI-FL

Many patients with GI-FL have been identified in the early stages, however, the disease lesion is not unifocal, but multifocal, with another lesion in the small intestine, and rituximab with or without chemotherapy for many non-advanced GI-FL cases may be initiated according to the regimens for nodal FL[24].

However, because it has been reported that there was

little difference in survival between GI-FL patients with and without therapies, and GI-FL itself is very rare, the management of GI-FL remains controversial at present[24].

In recent years as capsule endoscopy and double balloon endoscopy of the small intestine have been generalized in Japan, we have the opportunity to identify GI-FLs, particularly in patients with asymptomatic GI-FL. In addition, the therapeutic options in these patients such as whether conventional chemotherapy with rituximab should be initiated or no therapy with the "Watch and Wait" concept requires careful consideration. This decision may be very important.

Yamamoto *et al*[24] recently performed a literature review and reported the actual numbers in each therapeutic category. Observation without therapy was only about 25%, while some kind of therapy was performed in 75% of all primary GI-FL patients. Chemotherapy (or combination with other therapies) was performed in about 50% of all primary GI-FL patients.

Chemotherapy or other therapies tend to be performed more frequently in patients with GI-FL rather than observation without therapy based on the "Watch and Wait" concept. The reasons why GI-FL patients undergo chemotherapy are as follows: (1) because of increasing acceptability, generalization, dissemination, and recognition of the concepts of primary GI-FL and the chemotherapy regimens as treatment strategies for GI-FL; and (2) because it has been reported that the combination of conventional chemotherapy with rituximab can prolong the survival of nodal FL patients compared with conventional chemotherapy alone, rituximab-containing regimens have been administered to a growing number of GI-FL patients. Future prospective studies on the treatment of GI-FL patients are awaited with great interest.

CURRENT AND FUTURE DEVELOPMENTS

Therapeutic monoclonal antibodies, such as the anti-CD20 antibody, provide significant benefits to patients with NHL. In particular, since 2000 various phase Ⅲ trials have utilized RCTs to compare the treatment effects of conventional chemotherapies *vs* the addition of rituximab to these therapies. The results indicated that the combination of rituximab and conventional chemotherapy was generally superior both in terms of response rates and survival times. Thus, this combination therapy is gradually becoming the standard. Recently, phase Ⅰ and Ⅱ trial results assessing the initial use of a novel anti-CD2 antibody, alone or in combination with rituximab, in patients with recurrent aggressive lymphoma have been published. Determining the usefulness of these new antibodies compared to rituximab requires randomized comparative trials or a demonstration of their effectiveness in rituximab-refractory or relapsed patients after completion of first-line rituximab therapy.

Phase Ⅲ trials comparing galiximab with rituximab combined with galiximab are in progress. Similar studies will be necessary in the future to assess the effectiveness of many novel anti-CD20 antibodies. Some anticipate that rituximab, in combination with these new anti-CD20 antibodies, will improve response rates and prolong PFS.

Radioimmunotherapy with ^{90}Yttrium-ibritumomab tiuxetan or ^{131}Iodine-rituximab is safe and highly effective in patients with relapsed or refractory (resistant to rituximab-based therapy) FL. The effectiveness of these radiolabeled antibodies needs to be compared to other kinds of therapies in randomized trials.

Idiotype vaccines are immunotherapeutic products which have been developed to induce active and long-lasting immune responses against lymphoma cells. Most of these vaccines use the tumor B cell idiotype (the variable region of the surface immunoglobulin) as a tumor-specific antigen. Several phase Ⅰ / Ⅱ clinical trials of idiotype vaccines in FL have been conducted over the last 20 years, of which about 10 have been published and demonstrate the effectiveness of these vaccines. These encouraging results, which seem to indicate that the vaccines prolonged PFS, initiated three phase Ⅲ RCTs (Biovest, Genitope, and Favrille), however, all of these phase Ⅲ trials ultimately failed to prolong PFS.

Primary GI-FL is very rare, and this histology represents around 2% of all GI-NHL. Many patients with GI-FL have been found at the early stages, however, the disease lesion is not unifocal, but multifocal, with another lesion in the small intestine, and rituximab with or without chemotherapy for many non-advanced GI-FL cases may also be initiated based on the regimens for nodal FL. However, it has been reported that there was little difference in survival between GI-FL patients with and without therapies. Furthermore, GI-FL itself is very rare, and the management of GI-FL remains controversial at present. Yamamoto *et al*[24] recently performed a literature review and reported that only about 25% of primary GI-FL patients underwent observation without therapy, and chemotherapy (or combination with other therapies) was performed in about 50% of all primary GI-FL patients. Ongoing research on biomarkers to guide individualized GI-FL therapy may provide invaluable information which will lead to the establishment of a standard therapeutic regimen.

CONCLUSION

This article summarized the existing information on FL treatment through an extensive literature review which included the latest research on drug therapies. The various treatments reviewed included novel monoclonal antibodies, immunoradiotherapy, and the combination of these therapies with conventional chemotherapy. Moreover, idiotype vaccines, a nascent treatment alternative for which phase Ⅲ trials are now near completion, were discussed. If successful, the latter can potentially be developed as personalized anti-cancer treatments optimized for each patient. This article concluded with a brief discussion on potential future developments in the treatment of FL.

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TOPIC HIGHLIGHT

Marcela Kopacova, Associate Professor, MD, PhD, *Series Editor*

Technological advances in radiotherapy for esophageal cancer

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Abstract

Radiotherapy with concurrent chemotherapy and surgery represent the main treatment modalities in esophageal cancer. The goal of modern radiotherapy approaches, based on recent technological advances, is to minimize post-treatment complications by improving the gross tumor volume definition (positron emission tomography-based planning), reducing interfraction motion (image-guided radiotherapy) and intrafraction motion (respiratory-gated radiotherapy), and by better dose delivery to the precisely defined planning target volume (intensity-modulated radiotherapy and proton therapy). Reduction of radiotherapy-related toxicity is fundamental to the improvement of clinical results in esophageal cancer, although the dose escalation concept is controversial.

Key words: Esophageal cancer; Intensity-modulated radiotherapy; Positron emission tomography; Image-guided radiotherapy; Proton therapy

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INTRODUCTION

Despite many advances in diagnostics and multimodal treatment (surgery, radiotherapy, chemotherapy), esophageal cancer is still generally associated with poor prognosis. The resectability rate in esophageal cancer is reported to be 60%-90%, but the resulting 5-year overall survival rate of resectable disease ranges from 10%-25%[1].

The incidence rate of esophageal cancer varies considerably according to geographical location. The highest rates occur in northern China and northern Iran, where incidence exceeds 1 in 1000 individuals[1]. By contrast, the incidence of esophageal cancer in most European countries and United States does not exceed 1/10 000[2,3].

Histologically, the main tumor types are squamous cell carcinomas and adenocarcinomas. Adenocarcinomas usually occur in the lower third of the esophagus, and this histological type has greatly increased in the last decade in several european countries and in the United States[4].

Currently, radiotherapy has a well-defined role in the management of esophageal cancer. Together with surgery and chemotherapy, it represents the main treatment modality in esophageal cancer. However, recent technological advances in radiation treatment (e.g. intensity-modulated radiotherapy, image guided-radiotherapy, positron emission tomography based radiotherapy planning, *etc.*) have progressively changed the practice, particularly in esophageal cancer. The main goal of these new approaches is the precise irradiation of the tumor while minimizing the risk of damage to healthy tissues.

CURRENT STRATEGIES

Multidisciplinary strategies for treatment

The choice of treatment strategy depends primarily on the patient's performance status, stage and extent of the disease, histology, and location of the primary tumor. The main curative treatment modalities are surgery and concurrent radiotherapy and chemotherapy. In most resectable thoracic esophageal carcinomas (stage ⅡA-ⅣA), concurrent chemoradiotherapy is provided as a preoperative treatment or as definitive treatment[5]. Meta-analyses confirmed that preoperative chemoradiotherapy substantially downstages the tumor[6], and the combination of preoperative chemoradiotherapy and surgery significantly increases 3-year overall survival and reduces the local-regional recurrence rate compared to surgery alone[7]. A recent meta-analysis by Gebski *et al*[8] evaluated ten randomized trials of preoperative chemoradiotherapy compared with surgery alone and suggested a 13% absolute difference in survival at 2 years with the combined treatment. Definitive chemoradiotherapy is the preferred modality for cervical esophageal cancer[5].

Recently, perioperative chemotherapy ECF (combination of epirubicin, cisplatin, and 5-fluorouracil) has become available for adenocarcinomas of the lower esophagus and the gastroesophageal junction, based on the results of the MAGIC trial. This randomized trial compared perioperative chemotherapy plus surgery to surgery alone in patients with adenocarcinoma of the stomach (74%), gastroesophageal junction (11%), and lower esophagus (14%). The 5-year survival rates were 36.3% for the perioperative-chemotherapy group compared to 23.0% for the surgery alone group[9].

The current standard scheme of concurrent chemoradiotherapy is radiotherapy (RT) in a dose of 50-50.4 Gy in 5-5.5 wk and 5-fluorouracil (5-FU) and cisplatin (cDDP) based concurrent chemotherapy. This recommended scheme is based on the results of two randomized trials. RTOG 8501 compared chemoradiotherapy in a dose of 50 Gy plus cisplatin and 5-FU *vs* radiotherapy alone in a dose of 64 Gy. This trial demonstrated the survival advantage of combined treatment (26% *vs* 0% at 5 years, $P < 0.001$), despite the lower dose of radiotherapy[10]. The intergroup study 0123 (RTOG 94-05) compared two arms with the same concurrent chemotherapy regimen (cDDP, 5-FU) and radiotherapy in doses of 64.8 Gy *vs* 50.4 Gy. There was no benefit from the higher dose with respect to the local failure rate (56% *vs* 52% at 2 years) or overall survival (31% *vs* 40% at 2 years)[11].

Standard of radiotherapy planning

The current standard of radiation treatment is a three-dimensional conformal radiotherapy (3D-CRT) based on three-dimensional computer tomography (CT) planning, with volumes delineated according to the International Commision on Radiation Units and Measurements (ICRU) reports 50[12] and ICRU Supplement 62[13].

The gross tumor volume (GTV) is defined on CT slices as a macroscopic primary tumor and involved lymph nodes. Endoscopic evaluation, endoscopic ultrasonography (EUS), and/or barium swallow are helpful. The endoscopic marking of the upper and lower extension of the visible tumor with metallic clips improves the definition of the GTV[14].

The clinical target volume (CTV) includes the GTV and areas at risk of a microscopic spread of the disease. In general, it is recommended to use the cranial and caudal margin of 4 cm due to submucosal spread and 1 cm radially. The same margins were used in the latest Radiation Therapy Oncology Group trials for esophageal cancer[15]. The lymph nodes at risk are included in the CTV according to the location of the primary tumor. For instance, the supraclavicular lymph nodes are included in the CTV in cervical esophageal cancer[16]. The PTV includes the CTV plus a margin for internal movements, mainly respiratory movements, (internal margin - IM; internal target volume - ITV) and for setup uncertainties (setup margin - SM). A margin of 1-2 cm beyond the CTV is used[15].

Dosimetric parameters of toxicity

Modern radiotherapy approaches have to evaluate the probability of organ-specific radiation toxicity. Organs at risk for esophageal cancer radiotherapy include lungs, heart, spinal cord, and for distal esophageal cancer, the liver and kidneys. Current knowledge of radiation toxicity derives from conventional and 3D-CRT data.

Lung toxicity: The dosimetric parameters of lung injury risk were studied mainly on lung cancer irradiation, but a few studies evaluated the risk of lung toxicity in esophageal cancer. In these studies, the increased risk of radiation pneumonitis correlated with heterogeneous parameters, such as mean lung dose (D_{lung} mean), the percentage of lung volume receiving at least 20 Gy (V20), 13 Gy (V13), 10 Gy (V10) or 5 Gy (V5)[17-22].

Graham *et al*[17] found a strong correlation between parameter V20 and the severity of pneumonitis in lung cancer patients. They reported the incidence of grade ⩾ 2 pneumonitis as 7%, 13% and 36% for patients with V20 in the range of 22%-31%, 32%-40%, and > 40%, respectively. Kwa *et al*[18] considered the mean lung dose to

be the most useful predictor of radiation pneumonitis in thoracic tumor radiotherapy. Based on pooled data from 540 patients irradiated for thoracic malignancy, the calculated risk of grade ⩾ 2 pneumonitis was 43%, 18%, and 11% for mean lung doses of 24-36, 16-24 and 8-16 Gy, respectively. Schallenkamp *et al*[19], in a retrospective study, found the strongest predictors of pneumonitis to be the parameters V13 and V10. Lee *et al*[20] found, in 61 patients irradiated preoperatively with concurrent chemotherapy for esophageal cancer, a significant increase of postoperative pulmonary complications in cases with V10 > 40%. A more recent study from the same institution suggested that the factor most strongly associated with postoperative pulmonary complications is the volume of lung spared to doses of ⩾ 5 Gy[21]. Tucker *et al*[22] analyzed 110 patients with preoperative chemoradiotherapy for esophageal cancer and, in that cohort, the mean lung dose and absolute volume of lung volume receiving < 5 Gy were similar predictors of postoperative pulmonary complications.

Other potential risk factors for radiation pneumonitis, besides the heterogenous dosimetric parameters, have been proposed, such as concurrent chemotherapy, age, pretreatment pulmonary functions, presence of chronic pulmonary disease, and others[23-25]. Radiation oncologists have to judge all dosimetric and non-dosimetric risk factors for pulmonary complications of radiotherapy before approval of the treatment plan.

Heart toxicity: The most common manifestation of late radiation injury to the heart is pericardial disease. It may present as acute pericarditis, as chronic pericardial effusion, or can be asymptomatic. The myocardium is involved less frequently, but it develops into more serious cardiomyopathy, usually characterized pathologically as diffuse fibrosis. From long term surviving patients after radiotherapy for Hodgkin's lymphoma or left sided breast cancer, there is evidence that radiotherapy can substantially increase the risk of acute myocardial infarction after radiotherapy. The reason is probably an acceleration of coronary artery disease as a late effect of the radiotherapy[26].

Wei *et al*[27] evaluated the influence of definitive radiochemotherapy on pericardial toxicity. They reported, in 101 patients, a development of pericardial effusion in 27.7 % at a median of 5.3 mo. The pericardium volume irradiated by a dose of 30 Gy or higher (V30) was identified to be a significant predictor of pericardial effusion risk. Tripp *et al*[28] reported a significant reduction of ejection fraction in 20 patients after neoadjuvant chemoradiotherapy (59% *vs* 54 %, $P = 0.01$), but without significant clinical morbidity. Inferior left ventricle ischemia is commonly found in patients having received radiotherapy for distal esophageal cancer. The ischemic segments usually occurr in volumes irradiated to a dose of 45 Gy or more[29].

Spinal cord toxicity: The recommended maximum dose on the spinal cord is 45 Gy[5]. Current data suggests that the probability of myelopathy at 45 Gy is 0.03%, and at 50 Gy, is 0.2%[30]. In this case, the risk of radiation myelopathy in esophageal cancer is low, with a standard prescribed dose of 50.4 Gy as it is easy to comply to the recommended dose limits for the spinal cord. An increased risk of myelopathy can be expected in connection with a dose escalation approach.

Liver toxicity and kidney toxicity: The liver and kidneys are considered to be organs at risk mainly in distal esophageal cancer when the irradiated volume involves the upper abdomen. The risk of radiation injury is minimal when the mean liver dose is < 30 Gy[31] and if at least 50% of the functional kidney parenchyma is spared doses > 20 Gy, which is considered to be the tolerance dose for the human kidney[32].

NEW TECHNOLOGIES IN RADIOTHERAPY

Positron emission tomography/CT based radiation therapy planning

In esophageal cancer there is a high interobserver variability in target volume delineation among tumor sites[33]. The precise definition of the primary tumor and involved lymph nodes is crucial for RT planning.

Positron emission tomography (PET) with 18F-fluoro-2-deoxyglucose (FDG) as a tracer, was documented in several centres as a highly effective diagnostic modality for the initial staging in patients with esophageal cancer, especially in revealing lymph node involvement or distant metastases[34], although the sensitivity for regional lymph node involvement of FDG-PET is controversial[35-38]. FDG-PET imaging became beneficial in radiotherapy planning in several tumor types, mainly in lung cancer, because of the significant impact on target volume delineation[39]. Therefore, the concept of integration of PET into the RT planning process was rational.

Vrieze *et al*[40] added FDG-PET imaging to the RT planning process based on conventional CT and EUS status in 30 patients. In six patients, eight positive lymph nodes were identified on FDG-PET alone, but not on CT/EUS imaging. In three of these patients the target volumes would be enlarged. By contrast, in eight patients, nine positive lymph nodes were identified only on CT/EUS, but not on PET. Therefore, the authors recommended not to reduce the CTV based on negative lymph node FDG-PET status.

In another study, Konski *et al*[41] evaluated the impact of PET and EUS compared with CT alone in radiotherapy planning in 21 patients. Their results showed a low sensitivity of FDG-PET alone to determine regional lymph node metastases. EUS detected significantly more patients with periesophageal and celiac lymphadenopathy compared to PET and CT. Patients with periesophageal lymphadenopathy on PET had a higher primary tumor standard uptake value. The authors also found that the length of the primary tumor (GTV) was significantly longer when determined on CT scans compared with PET scans. The mean length of the GTV, as determined on PET, CT and endoscopy was 5.4, 6.77 and 5.1 cm, respectively.

The sensitivity of FDG-PET alone is considered by some authors to be low[37,38]. Compared to FDG-PET

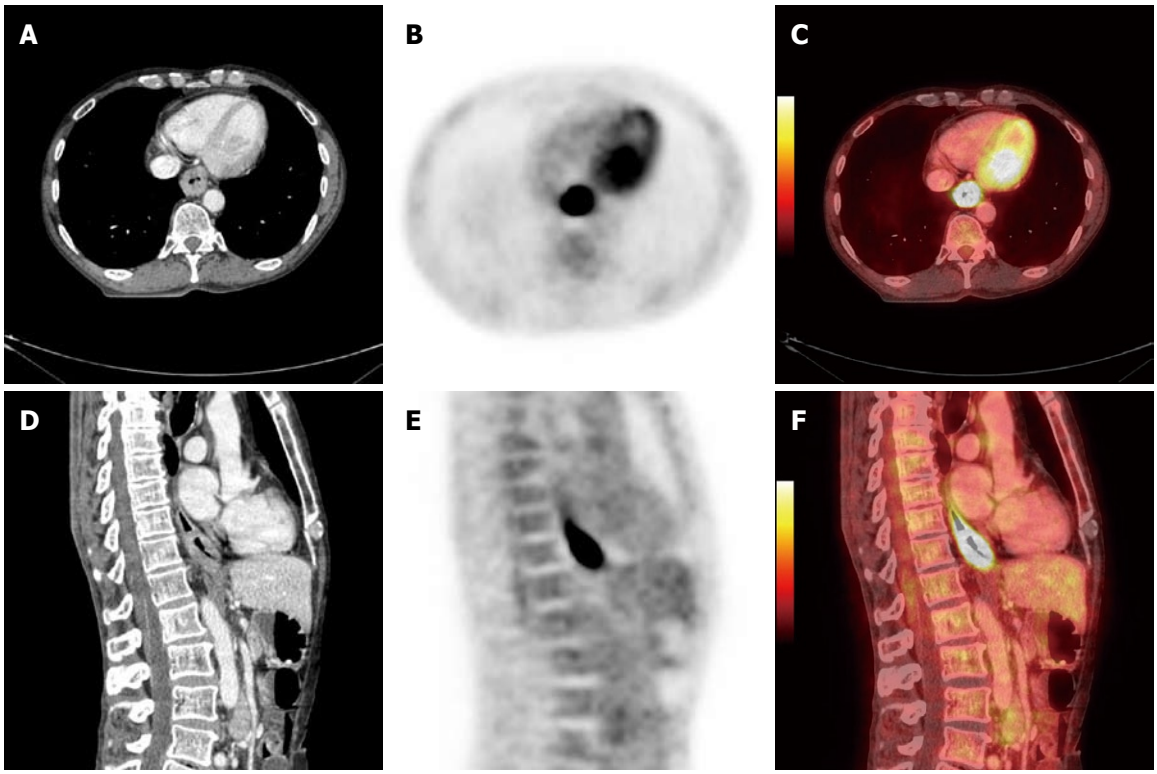

Figure 1 Positron emission tomography/computed tomography images of 61-year-old man with primary squamous cell carcinoma in distal part of the esophagus T3N0M0 prepared in treatment position for radiotherapy planning. A: Computed tomography (CT), axial slice; B: Positron emission tomography (PET), axial slice; C: PET/CT fusion, axial slice; D: CT, sagital slice; E: PET, sagital slice; F: PET/CT fusion, sagital slice.

alone, integrated PET/CT imaging improves the sensitivity and accuracy in the assessment of locoregional lymph nodes in esophageal cancer[42,43]. In a retrospective study by Muijs *et al*[44], target volumes were independently defined based on CT only and based on coregistered PET/CT in 21 patients. In that study, PET/CT-based planning of target volumes was inadequately covered by CT only-based treatment plans in eight patients (38%). In the study of Gondi *et al*[45], PET/CT-based target volumes were compared to CT only-based target volumes in 16 patients with esophageal cancer. In ten of these patients the addition of PET to the planning led to a reduction of the GTV volume.

Leong *et al*[46] evaluated the impact of PET/CT-based planning in a prospective trial. The target volumes based on CT alone and PET/CT were compared in 21 patients. The addition of PET information altered the clinical stage in eight patients (38%). Four patients had a distant metastatic disease and four had an unsuspected regional nodal disease. The PET findings led to a change in the management from radical chemoradiation to treatment with palliative intent in five of these patients (24%). In 16 patients (69%) the PET avid disease was not included in the GTV.

In a similar study by Moureau-Zabotto *et al*[47], the target volumes based on CT alone and subsequently on PET/CT were defined and compared in 34 patients. Unknown metastatic disease was detected by PET/CT in two patients. The GTV was modified in 19 patients (56%); in 12 of these patients the GTV was decreased and in seven patients it was increased by PET/CT. In 18 patients it led to the modification of the PTV. The influence of PET/CT based RT planning on the total lung volume receiving > 20 Gy was also shown.

Recently, Shimizu *et al*[48] published a study of 20 patients who were examined before surgery by PET/CT and EUS. Based on these preoperative diagnostic modalities, the target volumes for radiotherapy were defined and these volumes were compared to the histopathologic findings. Although hybrid PET/CT was used, the CTV was inadequately covered in seven cases out of 20. When EUS was added to PET/CT, inadequate CTV cover occurred in five cases compared to eight cases with CT only- based target volumes.

Therefore, despite the growing popularity of PET/CT based RT planning in esophageal cancer, the recently published International Atomic Energy Agency expert report 2006-2007 classifies esophageal cancer as a diagnosis where the use of PET or PET/CT for RT planning should be cautiously considered, as there is still limited supporting data[49].

An example of PET/CT images performed in treatment position for radiotherapy for esophageal cancer is shown in Figure 1.

Intensity-modulated radiation therapy

Intensity-modulated radiotherapy (IMRT) is an advanced form of conformal radiotherapy that utilizes computer-controlled linear accelerators to deliver precise radiation doses to the PTV (Figure 2). The principal of IMRT is the

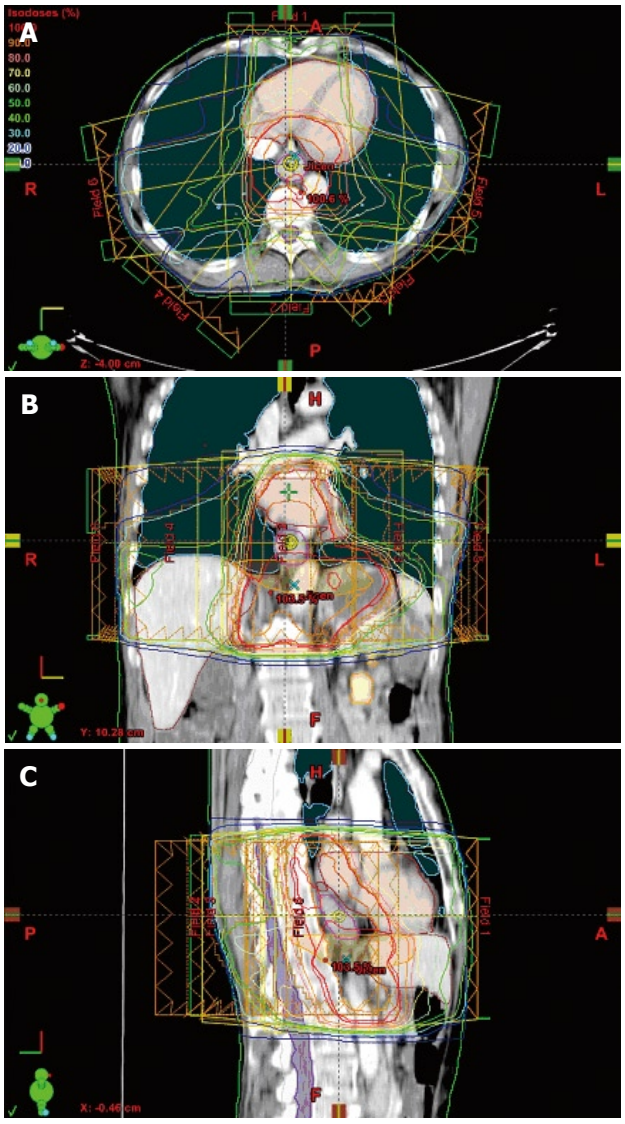

Figure 2 Intensity-modulated radiotherapy plan prepared on a positron emission tomography/computer tomography dataset (Figure 1). Delineated target volumes and organs at risk, beam arrangement and dose distribution in axial (A), coronal (B), and sagittal slices (C).

use of variable radiation fluence patterns from multiple beam angles. The beam fluences are calculated by automated computer-assisted optimization and the combination of optimal beam fluences results in an optimal dose distribution. Current commercial treatment planning software for optimization usually requires only a definition of beam configuration, PTV(s), and dose-volume constraints of organs at risk with varying penalty weights - so called inverse planning.

The main dosimetric advantage of IMRT is the possibility of better sparing of healthy tissues and organs at risk, including shape concavities of the PTV. Therefore, IMRT facilitates a lower risk of late adverse effects of radiotherapy and better local control of the tumor, due to the possibility of safe dose escalation. There has already been much data published worldwide demonstrating dosimetric, and even clinical, improvement of radiation treatment by IMRT, mainly in prostate cancer and head and neck cancer[50,51]. For these reasons, there has been a massive expansion of IMRT worldwide in the last few years.

In addition to the sparing of organs at risk and the possibility of dose escalation in the whole PTV, IMRT offers a dose escalation in every fraction in the subregion with a high risk of local recurrence (primary tumor or tumor bed). This principle is called simultaneous integrated boost (SIB), and it is used mainly in head and neck cancer. The advocates of SIB-IMRT techniques emphasize a better conformality of irradiation in comparison to shrinking volumes technique[52,53].

Several dosimetric studies have been published in esophageal cancer. Nutting *et al*[54] compared IMRT plans with various beam angles and 3D-CRT plans in five patients. They concluded that IMRT using conventional beam angles (two opposed antero-posterior fields and two posterior oblique fields) can provide an acceptable dose homogeneity within the PTV and reduces lung irradiation compared to 3D-CRT. The mean values of the mean lung dose and relative mean lung volume within a dose of 18 Gy were 11.0 Gy and 9.5 Gy (P = 0.001), and 18.8% and 14.1% (P = 0.001), for 4-field IMRT and 3D-CRT, respectively. IMRT plans using nine equispaced fields were not superior to 3D-CRT plan due to the increased lung volume involved with low doses of irradiation.

The reduction of lung dose by IMRT inverse planning in esophageal cancer was subsequently reported by Wu *et al*[55] They prepared IMRT plans using 3-9 equispaced beams and 3D-CRT plans in CT data of 15 patients. The percentage volume of the lung receiving 25 Gy or above (V25) was used as the main dosimetric parameter for the lungs. There was a considerable reduction in V25 in the IMRT plans compared with the inverse planned 3D-CRT (24.6% and 18.2% for the left and right lungs, respectively). The average mean heart dose was not statistically different, but the calculated normal tissue complications probability (NTCP) was significantly lower using IMRT.

The comparison of IMRT *vs* 3D-CRT plans in cervical esophageal cancer was the goal of the dosimetric study in five patients of Fenkell *et al*[56] IMRT plans provided better PTV coverage, higher conformality, a reduction of the maximum dose to the spinal cord and brain stem, and a lower mean dose to the parotid glands.

In another study for upper esophageal cancer, Fu *et al*[57] reported a dosimetric comparison of conformal radiotherapy and SIB-IMRT technique with 3, 5, 7 and 9 beams. The prescribed doses were 67.2 Gy and 50.4 Gy in the primary lesions and electively treated regions; the dose per fraction was 2.4 and 1.8 Gy, respectively. The authors concluded that IMRT-SIB shortens the total treatment time and the primary tumor can receive a higher equivalent dose by SIB. The five equispaced coplanar SIB-IMRT technique produced desirable dose distribution. This regimen is under clinical evaluation in this center.

Chandra *et al*[58] retrospectively compared 4-9 beam IMRT and 3D-CRT plans for distal esophageal cancer in ten patients. The IMRT improved PTV heterogeneity. There was a reduced total lung volume irradiated above 10 Gy and 20 Gy (V10 and V20), and the mean lung dose

using the IMRT plans. However, at low dose levels (below 7 Gy), there was a tendency of increased volume V7 with more beams for IMRT plans.

Mayo *et al*[59] described a technique of combined static and intensity-modulated beams for lung and esophageal cancer radiotherapy. This hybrid IMRT technique reduced the V5, V13, and V20 volumes in a subgroup of esophageal cancer compared to the IMRT only plans. The largest differences were in V13. However, there was an increase in dose to the heart in the hybrid IMRT plans.

The risk of larger volumes of lungs irradiated by lower doses of radiotherapy (⩾ 5 Gy; V5) was mentioned above. The IMRT plans can be associated with higher V5 because of the high number of beams and their configuration, but it can also be partially caused by a leakage of the multileaf collimator during IMRT[60].

Although the dosimetric studies demonstrated the advantage of IMRT in tumors of all parts of esophagus, the clinical results of IMRT for esophageal cancer are still limited to a few small studies. Wang *et al*[61] retrospectively analysed six patients treated by 5-9 beam IMRT with concurrent chemotherapy for locally advanced cervical and upper thoracic esophageal cancer. The prescription dose was 59.7-66 Gy in 28-33 fractions; in five patients a simultaneous integrated boost technique was used. In this study, all six patients achieved complete remission. However, two patients developed local recurrence; one patient had distant metastases and one patient had both regional and distant metastases. Three of the four surviving patients developed an esophageal stricture or fistula.

At Stanford University, 30 patients were treated with preoperative or definitive chemoradiotherapy using IMRT in a median dose of 50.4 Gy (range 34.2-58.8 Gy) for esophageal cancer. 67% of tumors were adenocarcinomas. The 2-year local-regional control was 83% in patients treated preoperatively and 51% in definitively treated patients. One patient died because of a complication following the placement of a gastrostomy tube during the irradiation course (after a dose 34.2 Gy). One patient developed postoperative acute respiratory distress syndrom. Eight patients (27%) developed late esophageal stricture requiring dilatations[62].

Tomotherapy

Helical tomotherapy is a form of intensity-modulated radiotherapy using a helical radiation delivery system. The beam delivery is similar to that of helical computed tomography.

Chen *et al*[63] compared three radiotherapy techniques diametrically - tomotherapy, step-and-shoot method of IMRT, and 3D-CRT - in the CT data of six patients. In the study, tomotherapy was superior to IMRT and 3D-CRT due to better dose conformity, dose homogeneity, and sparing of lung volume from doses ⩾ 20 Gy (V20). Helical tomotherapy and IMRT compared to 3D-CRT spared the heart better (decreased V30 and V45). However, tomotherapy and IMRT plans resulted in a larger V10 of lungs compared to 3D-CRT plans. The same centre also reported the first clinical results of tomotherapy with concurrent chemotherapy in 20 patients with esophageal cancer at the American Society for Radiation Oncology Annual Meeting 2007. The prescribed dose was 50 Gy in the GTV and 45 Gy in the region of a possible subclinical disease. Ten patients were indicated after chemoradiotherapy for surgery, and in eight of them, downstaging was noted with two complete responses. There was a clinically complete response in six patients without surgery. Grade 3 acute toxicity was noted in nine patients (45%) without any specification by the authors. No grade 4 toxicity occured. Two patients developed pneumonitis after surgery[64].

Image guided radiation therapy

The risk of set-up uncertaintities known from megavoltage portal imaging led to a development of modern technological devices integrated into linear accelerators facilitating high precision positioning of a patient before each irradiation (minimizing of interfraction movements). Image-guided radiotherapy (IGRT) generally means the process of two-dimensional or three-dimensional imaging of the patient in the treatment position on the linear accelerator before an irradiation, with the aim of minimizing of the set-up error. Currently, a wide variety of online 2D and 3D imaging is used - megavoltage portal imaging, kilovoltage imaging, kilovoltage CT (commonly cone-beam), megavoltage CT (mainly on helical tomotherapy), and others[65]. The IGRT software allows an image acquisition, matching with the referrence images and individual setup corrections.

The IGRT can be practiced as a daily procedure before each fraction of radiotherapy to minimize random and systemic errors[66]. The second strategy uses an evaluation of random setup errors and, based on the result, the PTV is corrected. This concept, described by Yan *et al*[67,68], was first implemented in prostate cancer radiotherapy and was called by the authors *adaptive radiotherapy*.

Stroom *et al*[69] recommended the size of the CTV-PTV margin which ensures at least 95% of the prescribed dose to 99% of the CTV, to be equal to about $2\Sigma + 0.7\sigma$, where Σ is the standard deviation of the distribution of systemic deviations and σ is the average standard deviation of the distribution of random deviations. Chen *et al*[70] calculated these parameters in ten patients who underwent helical tomotherapy for esophageal cancer. The suggested CTV-PTV margins were 5.0 mm in the anterior-posterior direction, 11.1 mm in the lateral direction and 12.7 mm in the superior-inferior direction. Therefore, the authors recommend a megavoltage CT on tomotherapy before each fraction to minimize setup errors when lesser CTV-PTV margins are used.

Hawkins *et al*[71] recently presented the concept of cone-beam CT-derived adaptive radiotherapy for esophageal cancer treatment at their center. In 14 cases, the standard plan with CTV-PTV margin of 1 cm was prepared. The cone-beam CT was obtained before the first four fractions and, based on this data, the composite CTV with 5 mm margin for the PTV was defined. The same process was subsequently repeated weekly and the plans were compared. The study demonstrated a significant reduction

in the dose received by the heart and lungs because of lesser individualized CTV-PTV margins.

4D-computer tomography and respiratory control techniques

The limitation of the precise dose delivery by 3D-CRT or IMRT in the region of the thorax and upper abdomen are physiologic movements of tumor and organs, mostly due to respiratory or cardiac cycles (intrafraction movements). Therefore, radiation oncologists have to estimate the internal target margin to be adequate to these movements to cover target volumes sufficiently.

The respiratory motion of anatomic structures and target volumes has been investigated in several diagnoses, mainly in lung cancer, breast cancer, and upper abdomen malignancies. Currently, the optimal method for acquiring exact information on movements of structures during the respiratory cycle is respiratory-correlated computed tomography imaging (4D-CT). This technology allows the capture of CT data in separate phases of the respiratory cycle. Their co-registration gives precise information on the amplitude of the structure motion and the position of the structure in each phase of the cycle.

New technologies were evolved to compensate for tumor motion to lower the internal margin for the PTV and, hence, to reduce the volume of surrounding healthy tissues. These include active or passive respiratory gating (respiratory gated radiotherapy) and respiratory tracking techniques.

The principle of active breathing control (ABC) is the monitoring of the respiratory cycle by a special mouth apparatus measuring the airflow (spirometric system). At a preset phase of the cycle (preset volume of expired air), the ABC apparatus temporarily blocks the airflow of the patient and during this period the irradiation is applied[72]. The Real-Time Position Management™ (RPM) System (Varian Medical System, Palo Alto, USA) uses infrared camera monitoring of the movements of a small plastic box with reflective markers placed on the skin in the patient's upper abdomen during free breathing or active breathing.

Real-time respiratory tracking is a method that dynamically moves or shapes the radiation beam to follow the tumor's motion during irradiation. This method was first evolved for CyberKnife System (Accuray, Inc., Sunnyvale, USA), which features a linear accelerator mounted on a robotic arm (Synchrony Respiratory Tracking System).

The evaluation of esophageal tumor movements during the respiratory cycle was studied by Lorchel *et al*[73] in eight patients with various locations of the primary tumor in the esophagus. The cumulative distribution of the GTV and CTV motion in all three directions in absolute terms showed that 95% of the data ranged from 0 to 0.8 cm and from 0 to 1.0 cm, respectively. Therefore, the authors recommended the size of the internal target margin of 1 cm. Zhao *et al*[74] quantified the internal target motion in tumors near the gastroesophageal junction. They found that the tumors exhibited asymmetric and directional changes in shape and volume, mainly for large GTVs. The mean range of the aboral margin of the tumor was greatest in the caudal direction at 0.91 ± 0.36 cm. The mean range of the motion in anterior and posterior directions was 0.68 ± 0.23 cm and 0.36 ± 0.13 cm, respectively. The lateral motion was 0.27 ± 0.09 cm to the right and 0.63 ± 0.20 cm to the left because of movements of the stomach on the left side of the GTV. Based on these results, the authors suggested the use of asymmetric internal target margins: 1.0 cm to the left, 0.8 cm to the right, 1.1 cm anteriorly, 0.6 cm posteriorly, 1.0 cm superiorly, and 1.6 cm inferiorly. The study also evaluated the influence of the heart beat: the largest mean range of motion was 0.56 ± 0.18 cm for the esophageal wall adjacent to the heart.

The optimal approach to respiratory gated radiotherapy was studied by Lorchel *et al*[75]. In a dosimetric study, four spiral scans were performed in eight patients with advanced esophageal cancer: one in the end of expiration, one in the end of inspiration, one in deep inspiration breathhold, and one acquisition was performed in free breathing. Based on the results, the authors suggested the irradiation of esophageal cancer patients in deep inspiration breathhold in the case of using spirometric system and in the inspiration phase in the case of free breathing gating system to reduce a dose to the lungs (reduction in V20) and heart (decreased V40).

Proton therapy

The idea of using proton beam therapy as an effective radiotherapy method was first proposed by Wilson *et al*[76] in 1946. The main advantage of proton radiotherapy is a better dose conformity due to proton beam characteristics. Proton beams are characterized by a narrow penumbra and maximal energy transfer at the end of the range in tissue (Bragg peak). The proton therapy of various malignancies has been discussed in recent years. A few studies on proton therapy for esophageal cancer are also available[77-80].

The dosimetric study of Isacsson *et al*[77] compared the treatment plans with proton beams to plans with photons and combined plans in five patients with esophageal cancer. There was an evident advantage of proton plans in the reduction of dose to organs at risk in all patients. The sparing of the lungs using proton radiotherapy compared to IMRT was noted by Zhang *et al*[78] in a dosimetric study for distal esophageal cancer.

The published clinical studies using proton beam therapy for esophageal cancer applied a dose escalation approach. Koyama *et al*[79] irradiated 30 patients (13 patients with superficial and 17 patients with advanced tumors) with proton beam therapy alone (median fraction dose of 3.2 Gy) or in combination with photons (median fraction dose of 3.1 Gy). Overall mean total doses were 77.7 Gy in superficial carcinomas and 80.7 Gy in advanced carcinomas, respectively. The rates of local recurrence at 5 and 10 years were 0% for superficial cancer, and 56.6% and 78.3%, respectively, for advanced cancer. The radiation-induced esophageal ulcer as a late effect occurred in 66.7% of patients.

Sugahara *et al*[80] reported the clinical results of proton

beam therapy in 46 patients. Forty patients were treated by a combination of photons and protons as a boost to a median total dose of 76.0 Gy (median fraction dose 3.0 Gy), six patients were irradiated only by protons to a median total dose of 82.0 Gy (median fraction dose 3.1 Gy). The local control at 5 years was 57% (83% with stage T1 and 29% with stage T2-T4). Postradiation esophageal ulcers were developed in 48% of patients.

CONCLUSION

The main aim of modern radiotherapy approaches in esophageal cancer is to minimize the post-treatment complications by the improvement of the GTV definition (PET-based planning), reduced interfraction motion (image-guided RT) and intrafraction motion (respiratory-gated RT), and better delivery of the dose to the precisely defined PTV (intensity-modulated RT and proton RT). Another systematic review by Yang *et al*[81] therefore encouraged to utilize and study all technological advances to maximize outcomes in esophageal and gastroesophageal junction cancers.

The reduction of radiotherapy-related toxicity by modern radiation techniques for esophageal cancer is fundamental to the improvement of clinical results of radiotherapy for esophageal cancer, although the dose escalation concept is still controversial.

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ORIGINAL ARTICLE

Chronic stress sensitizes rats to pancreatitis induced by cerulein: Role of TNF-α

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Abstract

AIM: To investigate chronic stress as a susceptibility factor for developing pancreatitis, as well as tumor necrosis factor-α (TNF-α) as a putative sensitizer.

METHODS: Rat pancreatic acini were used to analyze the influence of TNF-α on submaximal (50 pmol/L) cholecystokinin (CCK) stimulation. Chronic restraint (4 h every day for 21 d) was used to evaluate the effects of submaximal (0.2 μg/kg per hour) cerulein stimulation on chronically stressed rats.

RESULTS: *In vitro* exposure of pancreatic acini to TNF-α disorganized the actin cytoskeleton. This was further increased by TNF-α/CCK treatment, which additionally reduced amylase secretion, and increased trypsin and nuclear factor-κB activities in a protein-kinase-C δ and ε-dependent manner. TNF-α/CCK also enhanced caspases' activity and lactate dehydrogenase release, induced ATP loss, and augmented the ADP/ATP ratio. *In vivo*, rats under chronic restraint exhibited elevated serum and pancreatic TNF-α levels. Serum, pancreatic, and lung inflammatory parameters, as well as caspases' activity in pancreatic and lung tissue, were substantially enhanced in stressed/cerulein-treated rats, which also experienced tissues' ATP loss and greater ADP/ATP ratios. Histological examination revealed that stressed/cerulein-treated animals developed abundant pancreatic and lung edema, hemorrhage and leukocyte infiltrate, and pancreatic necrosis. Pancreatitis severity was greatly decreased by treating animals with an anti-TNF-α-antibody, which diminished all inflammatory parameters, histopathological scores, and apoptotic/necrotic markers in stressed/cerulein-treated rats.

CONCLUSION: In rats, chronic stress increases susceptibility for developing pancreatitis, which involves TNF-α sensitization of pancreatic acinar cells to undergo injury by physiological cerulein stimulation.

Key words: Pancreatitis; Stress; Tumor necrosis factor-α

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INTRODUCTION

Stress can be defined as "threatened homeostasis". Stressors can include physical or mental forces, or combinations of both. The reaction of an individual to a given stressor involves the stimulation of pathways within the brain leading to activation of the hypothalamic-pituitary-adrenal axis and the central sympathetic outflow[1]. These can result in visceral hypersensitivity through the release of different substances, such as substance P and calcitonin gene-related peptide from afferent nerve fibers[2].

While it is well established that a previous acute-short-term stress decreases the degree of severity of pancreatitis in several experimental models[3,4], the effects of chronic stress on the exocrine pancreas have received relatively little attention[5,6]. Chronic stress has been proved to increase the susceptibility of different rat organs, such as the small intestine, colon, and brain, to inflammatory diseases[2,7-9], as well as to aggravate atherosclerotic lesions in mice[10].

The pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α), has an important role in various biological functions, including cell proliferation, cell differentiation, survival, apoptosis and necrosis[11], and in stress-related inflammatory disorders[8-10,12]. Secretion of TNF-α by several stressful stimuli has been demonstrated in many cell types, including pancreatic acinar cells[13-20]. Previous reports evaluated the response of pancreatic acinar cells to exogenous TNF-α, showing disruption of the actin cytoskeleton and activation of nuclear factor-κB (NF-κB), a key transcriptional regulator of the expression of inflammatory molecules[21-23]. Interestingly, this TNF-α activation of NF-κB is mediated by the novel protein kinase C δ (PKCδ) and PKCε[23], which have also been shown to modulate cerulein-induced zymogen activation in these cells[24]. Although TNF-α has been shown to participate in the inflammatory cascade that propagates pancreatitis[25], its relevance in the genesis of this multifactor disease was hardly investigated.

We examined the possibility that chronic stress is a susceptibility factor for developing pancreatitis and that TNF-α is a putative sensitizer. We performed studies on both *in vitro* dispersed rat pancreatic acini stimulated with TNF-α and submaximal doses of cholecystokinin (CCK), and in *in vivo* rats under chronic stress induced by restraint and challenged with submaximal doses of the CCK-analogue cerulein.

MATERIALS AND METHODS

Antibodies and reagents

Antibodies used include those against rat TNF-α (R&D Systems, Minneapolis, MN), Na^+/K^+ ATPase (Upstate Biotechnology-Chemicon Int., Temecula, CA), PKCδ and PKCε (Santa Cruz Biotechnology, Santa Cruz, CA), and tubulin (Sigma Chemical Co., St. Louis, MO). Alexa Fluor 488-phalloidin was from Molecular Probes (Burlington, ON, Canada). Recombinant TNF-α was from R&D Systems. Sulfated CCK octapeptide, cerulein, Hoechst dye 33258, N-acetyl-cysteine (NAC), hexadecyltrimethylammonium bromide, and o-dianisidine hydrochloride were from Sigma Chemical Co. Calphostin C, and Gö6976 were from Calbiochem (San Diego, CA). PKCζ myristolated pseudosubstrate inhibitor was from Biosource International (Camarillo, CA). The PKCδ-specific antagonist peptide δV1-1was synthesized by American Peptide Company, Inc. (Sunnyvale, CA) and conjugated to a TAT peptide (PKCδ translocation inhibitor δV1-1). PKCε translocation inhibitor εV1-2 was from Anaspec (San Jose, CA). The protease inhibitor cocktail was from BD Pharmingen (San Jose, CA). BoC-Glu-Ala-Arg-MCA was from Peptides International (Louisville, KY). Amylase, lactate dehydrogenase (LDH), glucose, urea, creatinine, calcium, total proteins, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and quantitative C-reactive protein assay kits were from Wiener Lab (Rosario, Santa Fe, Argentina). The lipase assay kit was from Randox Laboratories Ltd. (Antrim, UK). NF-κB (p65), NF-κB (p50) transcription factor, lipid hydroperoxide (LPO), and malondialdehyde (MDA) Assay Kits were from Cayman Chemical Company (Ann Arbor, MI). TNF-α and macrophage inflammatory protein-1α (MIP-1α) Kits were from R&D Systems. The hypoxia inducible factor-1α (HIF-1α) Kit was from Genxbio Health Science (Delhi, India). The protein assay kit was from Bio-Rad (Hercules, CA). The heat shock protein (HSP) 72 Kit was from StressGen Biotechnologies, San Diego, CA). PKCδ and PKCε Kinase Assay Kits were from Cell Signaling Technology (Beverly, MA). Interleukin (IL)-6 and IL-10 Kits were from Assay Designs (Ann Arbor, MI). The CasPASE Apoptosis Activity Assay Kit was from Genotech (Maryland Heights, MO). The Enliten ATP Assay Kit was from Promega (Madison, WI). The ApoSensor ADP/ATP Ratio Assay Kit was from Enzo Life Science International Inc. (Plymouth Meeting, PA).

Animal model

Male Wistar rats (200-250 g) were housed in standard cages in a climate-controlled room with a temperature of 23 ± 2℃ and a 12 h light/dark cycle (lights on at 08.00 h), with free access to food and water except during restraint times. All animals were maintained under constant conditions for 4 d prior to stress, and they were randomly assigned to non-stress (control) or stress groups. Every day at 09.00 h, animals from both non-stress and stress groups received either 50 μg/kg TNF-α-neutralizing antibodies or an equal dose of control IgG, given as intraperitoneal (ip) injections. Rats in the stress group were exposed to various sessions of restraint (4 h every day for 21 d) between 10.00 and 14.00 h in the animal homeroom. The immobilization was performed using a metallic restraint jacket and was placed inside their home cage during the restraint sessions. Control rats were handled once at 10.00 h

for few seconds and left undisturbed in their home cages. Food and water were removed from their cages to avoid their interference in the parameters determined. Following the 21 d stress protocol, rats from each condition, non-stress (-) and stress (+), were then randomly distributed into three groups of four rats each. One group was treated with six doses of saline-vehicle (Veh groups), and the other two groups were treated with six doses of 0.2 μg/kg cerulein (Cer groups and anti-TNF-α plus Cer groups), given as hourly ip injections. Rats were sacrificed 1 h after the last injection by decapitation, and blood was drained onto heparinized dishes for white blood cell count and determination of hematocrit. Amylase (end-point colorimetric method), lipase (UV turbidimetric method), TNF-α [enzyme-linked immunosorbent assay (ELISA)], LDH (German Society of Clinical Chemistry-DGKC optimized kinetic method), glucose (enzymatic method), urea (enzymatic method), creatinine (end-point colorimetric method), calcium (direct colorimetric method), total proteins (enzymatic method), AST and ALT (IFCC optimized UV method), C-reactive protein (immunoturbidimetric method), HSP-72 (ELISA assay), IL-6 (ELISA assay), IL-10 (ELISA assay), and MIP-1α (ELISA assay) levels were assessed in serum using the respective assay kits. Pancreatic and lung tissues were obtained for determination of levels of NF-κB (p65) and NF-κB (p50) in nuclear extracts, and TNF-α using the respective ELISA assay kit, and myeloperoxidase (MPO) activity. Pancreatic tissue was also evaluated for levels of HIF-1α (ELISA assay), LPO and MDA (colorimetric method) using the respective assay kit, and trypsin activity. Caspases 2, 3, 8, and 9 activity (colorimetric/fluorometric method), and ADP and ATP levels (bioluminescent method) were determined in pancreatic, lung, and stomach tissue using the respective assay kits. All kits were used according to the manufacturer's instructions. All experimental protocols were approved by the Animal Care and Use Committee of the CBRHC Research Center.

Dispersed acini preparation

The preparation of isolated pancreatic acini from rats was performed by a mechanical and enzymatic dissociation technique[26]. The acini were resuspended in oxygenated Krebs-Ringer-HEPES (KRH) buffer, consisting of (in mmol/L): 104 NaCl, 5 KCl, 1 KH_2PO_4, 1.2 $MgCl_2$, 2 $CaCl_2$, 0.2% (wt/vol) bovine serum albumin, 0.01% (wt/vol) soybean trypsin inhibitor, 10 glucose, and 25 2-hydroxyethylpiperazine-2-ethanesulfonic acid (HEPES)/NaOH, pH 7.4, supplemented with minimal essential and non-essential amino acid solution and glutamine.

Cellular models

Hypoxia and reoxygenation treatment: Isolated pancreatic acini were seeded in six-well plates. The hypoxia and reoxygenation (H/R) media were produced by equilibrating Krebs-Henseleit buffer, consisting of (in mmol/L): 118.3 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25.0 $NaHCO_3$, and 11.0 glucose, pH 7.4, in 95% N_2/5% CO_2 or 95% air/5% CO_2, respectively. The studies were carried out using two water-jacketed incubators at 37℃ as follows. For H/R treatment, acini were exposed to an anoxic gas mixture (95% N_2/5% CO_2) in a humidified incubator for 30 min. After the hypoxia period, hypoxic media was rapidly replaced with reoxygenation media and the acini were transferred to 95% air/5% CO_2 in a humidified incubator for 3 h. For normoxia (N) control treatment, acini were exposed to 95% air/5% CO_2 in a humidified incubator for the same period of time as the H/R samples. To determine the effect of reactive oxygen species (ROS) inhibition, NAC (30 mmol/L) was added to the cells 1 h before hypoxia, and it was maintained in the media throughout the experiment. TNF-α released into the supernatant was determined using an ELISA assay kit according to the manufacturer's instructions. Values were normalized by DNA content in each sample, which was measured using Hoechst dye 33258.

Hydrogen peroxide stimulation: Isolated pancreatic acini were seeded in six-well plates and incubated with hydrogen peroxide (H_2O_2, 50 μmol/L) for 3 h at 37℃ in a 5% CO_2 humidified atmosphere. To determine the effect of ROS inhibition, NAC (30 mmol/L) was added to the cells 1 h before H_2O_2, and it was maintained in the media throughout the experiment. TNF-α released into the supernatant was determined by the corresponding ELISA assay kit and values were normalized by DNA content in each sample.

TNF-α and CCK stimulation: Isolated pancreatic acini were stimulated with CCK (50 pmol/L) for 1 h at 37℃. Pretreatment with the indicated concentrations of TNF-α in KRH was performed for 1 h prior to and along stimulation. Levels of amylase and LDH release, NF-κB (p65) and NF-κB (p50) in nuclear extracts, caspases 2, 3, 8, and 9 activity, and ADP and ATP, were measured in pancreatic acini using the respective assay kits, according to the manufacturer's instructions. Acini were also assessed for trypsin activity.

Amylase secretion: Amylase released into the supernatant and amylase content of the acini pellet were determined by a colorimetric method as previously reported[27]. "Total amylase" is defined as the summation of the amylase content in the respective cell pellet plus supernatant, and the amylase secreted into the supernatant is expressed as a percentage of total amylase.

Confocal immunofluorescence microscopy: Dispersed acini were placed on glass coverslips and after indicated treatments fixed with 4% paraformaldehyde. Fixed coverslips were permeabilized with 0.1% Triton X-100 for 15 min, followed by incubation with 1% bovine serum albumin for 1 h. Acini were then incubated with Alexa Fluor 488-phalloidin (1:500) for 1 h at 25℃. Coverslips were examined with a 63 × oil immersion objective and conventional laser excitation and filter set, by a laser scanning

confocal imaging system (Zeiss LSM510) equipped with the LSM software version 5.00 (Carl Zeiss, Oberkochen, Germany).

Subcellular fractionation and immunoblotting

Subcellular fractionation: Isolated pancreatic acini, after equilibration (20 min, 37℃), were subjected to the indicated stimulation, and then terminated by adding an excess volume of ice-cold KRH buffer. The acini were then pelleted by centrifugation (300 *g*, 4℃). Whole cell lysates and purified membranes were prepared from the treated dispersed acini by sucrose density gradient centrifugation. Briefly, the sucrose buffer consisted of: 0.3 mol/L sucrose, 0.01% soybean trypsin inhibitor, 0.5 mol/L phenylmethanesulfonyl fluoride, and 5 mmol/L β-mercaptoethanol. The acini were homogenized in a Potter-Elvehjem homogenizer, followed by a five min centrifugation (14 000 *g*, 4℃) to separate the nuclei pellets from supernatants. The supernatant fractions were centrifuged (15 min, 14 000 *g*, 4℃) to separate the zymogen granule pellets, and the resulting supernatants subjected to ultracentrifugation (3 h, 93 000 *g*, 4℃) to obtain the membrane pellet and cytosol supernatant fractions.

Immunoblotting: The protein contents of all samples were determined by Bradford method. Samples of subcellular fractions were dissolved in Laemmli buffer and boiled for five min. Equal amounts of protein were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Blots were blocked for 1 h in Tris-buffered saline containing 5% bovine serum albumin and then incubated with the corresponding primary antibody. The bound antibody was visualized by relevant peroxidase-coupled secondary antibodies using the enhanced chemiluminescence method (Amersham, Arlington Heights, IL). Quantification was performed by densitometry using the NIH-Image software.

Isoform-specific PKC activity

PKCδ and ε activities were determined on lysates from isolated acinar cells using the respective ELISA assay kit, according to the manufacturer's instructions. Activity values were normalized to the basal activity in non-stimulated control cells.

Trypsin activity

Pancreatic tissue and pancreatic acini samples were pelleted, and the cell pellet was resuspended in ice-cold morpholino propylsulfonate (MOPS) buffer, pH 7.0 (containing in mmol/L: 250 sucrose, 5 MOPS, and 1 $MgSO_4$), and then homogenized by hand using a Teflon and glass homogenizer. The resulting homogenate was centrifuged, and the supernatant was used for the assay. Trypsin activity was measured fluorometrically in a stirred cuvette at 37℃ in a Hitachi F-2000 spectrofluorometer with excitation at 380 nm and emission at 440 nm, using BoC-Glu-Ala-Arg-MCA as the substrate. Briefly, the slope of rising fluorescence emission was calculated as arbitrary units and normalized per DNA content in the homogenate of each sample.

Caspases' activity

Caspase 2, 3, 8, and 9 were measured using a colorimetric/fluorometric method as previously reported[28]. Briefly, pancreatic, lung or stomach tissue, or pancreatic acinar cell samples were homogenized in chilled lysis buffer and the lysates were centrifuged at 15 000 *g* for 30 min at 4℃. Caspases' activity was assessed on the supernatants.

ATP and ADP levels

ATP and ADP were measured using a bioluminescent method, as previously reported[28]. Briefly, pancreatic, lung, or stomach tissue, or pancreatic acinar cell samples were homogenized in 6% TCA (trichloroacetic acid) for one min and centrifuged at 6000 *g* for 5 min at 4℃. The TCA in the supernatant was neutralized and diluted to a final concentration of 0.1% with Tris-Acetate buffer, pH 7.75. ATP and ADP were measured in the supernatant. ATP loss was calculated as a percentage of the decrease in values corresponding to control.

MPO activity

Pancreatic and lung tissue were analyzed for MPO activity. Briefly, approximately 50 mg of pancreatic or lung tissue was homogenized on ice with 1 mL of 0.5% hexadecyltrimethylammonium bromide in 50 mmol/L phosphate buffer-pH 6.0. The homogenate was sonicated for 10 s at 200 W, and then centrifuged at 40 000 *g* for 15 min at 4℃. MPO activity in the supernatant was assayed as follows: 0.1 mL of supernatant was combined with 2.9 mL of 5 mmol/L phosphate buffer pH 6.0, 0.167 mg/mL o-dianisidine hydrochloride, and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured spectrophotometrically. One unit of MPO activity is defined as the amount that degrades 1 μmol of peroxide per minute at 25℃. MPO activity was normalized by DNA content in the homogenate of each sample.

Extraction of DNA

The total mass of DNA in the homogenate of each sample was used to standardize the units of enzymatic activity. Approximately 50 mg of tissue was homogenized in 400 μL of lysis buffer consisting of 0.5% SDS, 0.1 mol/L NaCl, 50 mmol/L Tris pH 8, 2.5 mmol/L EDTA, and 100 μg/mL proteinase K, for 1 h at 63℃ and 650 r/min. Seventy-five microliters of 8 mol/L potassium acetate followed by 500 μL of chloroform were then added. Samples were frozen at -20℃ for 15 min and centrifuged at 10 000 r/min for 5 min at 4℃. The DNA was extracted in 1 mL of absolute ethanol and washed in 1 mL of 70% ethanol. The clean DNA pellet was resuspended in distilled water buffer and DNA was measured at 260 nm.

Histological evaluation

Pancreatic, lung, and stomach tissues were graded on a scale of 0 to 4 each for edema, hemorrhage, leukocyte in-

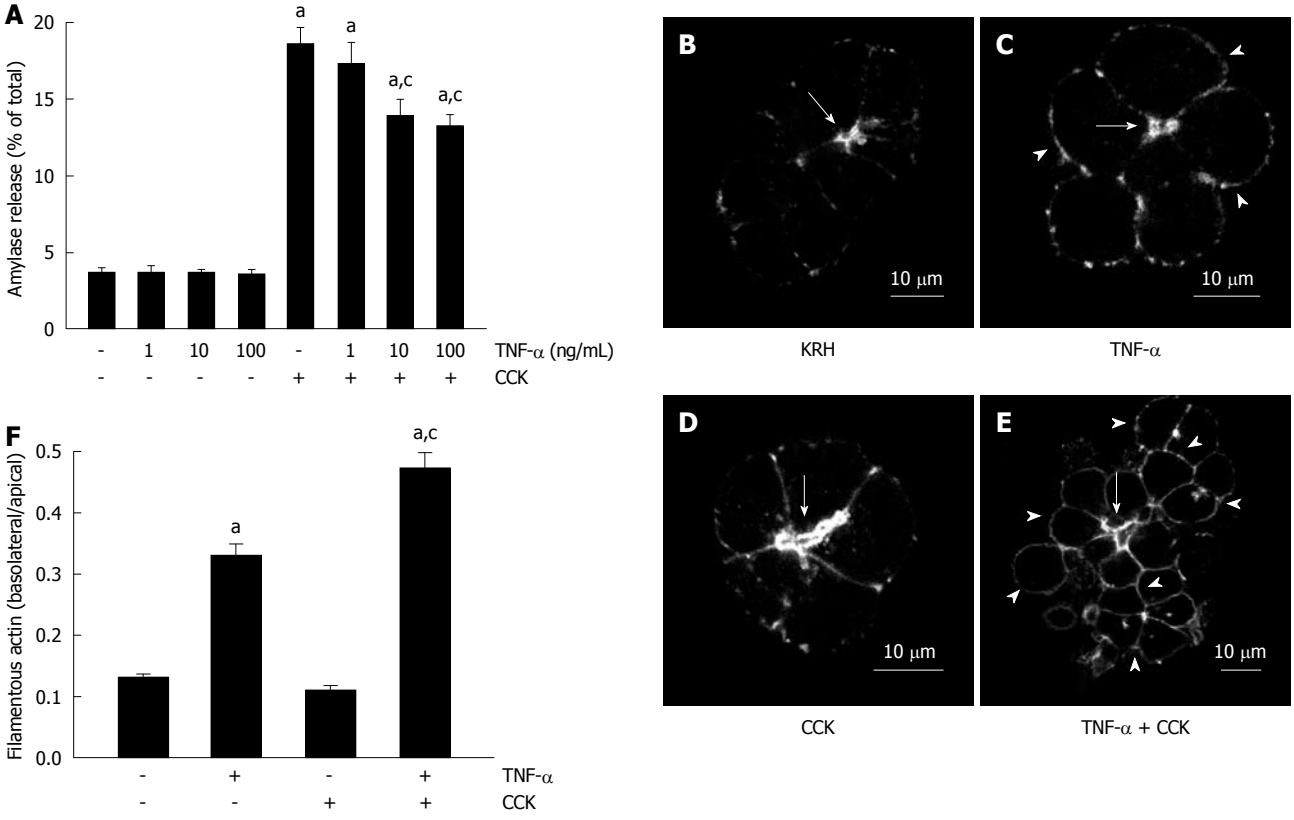

Figure 1 Tumor necrosis factor-α disorganizes the actin cytoskeleton and reduces submaximal cholecystokinin-stimulated amylase secretion. A: Amylase secretion from pancreatic acini. Isolated pancreatic acini were stimulated with either the indicated concentrations of tumor necrosis factor-α (TNF-α) for 2 h, or 50 pmol/L cholecystokinin (CCK) for 1 h, or the indicated concentrations of TNF-α for 1 h followed by the indicated concentrations of TNF-α plus 50 pmol/L CCK for 1 h, or Krebs-Ringer-HEPES (KRH) (vehicle-control) for 2 h. Amylase secreted into the media was determined and expressed as a percentage of the total cellular amylase of the respective sample. Results correspond to the mean ± SE from four independent experiments, with samples performed in triplicate. $^{a}P < 0.05$ *vs* KRH; $^{c}P < 0.05$ *vs* CCK alone; B-F: Filamentous actin distribution; B-E: Confocal images of filamentous actin localization in pancreatic acinar cells. Isolated pancreatic acini were treated with either KRH for 2 h (B), or 10 ng/mL TNF-α for 2 h (C), or 50 pmol/L CCK for 1 h (D), or 10 ng/mL TNF-α for 1 h followed by 10 ng/mL TNF-α plus 50 pmol/L CCK for 1 h (E). Fixed and permeabilized acini were stained with Alexa Fluor 488-phalloidin. Arrows show apical lumens, and arrowheads show basolateral membranes. These are representative images selected from three independent experiments, with samples performed in triplicate; F: Quantification of filamentous actin distribution. Areas of the apical and basolateral membranes in acinar cells treated as in B-E were delineated and the fluorescence intensity was determined. Sixty cells from three independent experiments were analyzed per condition. Values correspond to the intensity in the basolateral area *vs* the apical area, and are expressed as the mean ± SE. $^{a}P < 0.05$ *vs* KRH; $^{c}P < 0.05$ *vs* TNF-α alone.

filtrate, and necrosis. Histological changes were scored in a blinded manner by two independent observers counting frequency of foci per field seen at 40 × (absent, 0; mild, 1; moderate, 2; severe, 3; overwhelming, 4).

Statistical analysis

All data are presented as mean ± SE. The data for each group were subject to analysis of variance followed by Dunnet post hoc test when comparing three or more groups, or evaluated using Student's *t*-test when comparing only two groups. Significant differences were considered with values of $P < 0.05$.

RESULTS

TNF-α reduces submaximal CCK-stimulated amylase secretion

We first examined the effects of TNF-α on amylase secretion from dispersed rat pancreatic acini. Different samples were incubated with different concentrations of TNF-α. Figure 1A shows that none of them stimulated amylase release (3.6% ± 0.5%, 3.5% ± 0.4% and 3.4% ± 0.5% for 1, 10 and 100 ng/mL, respectively) over basal vehicle-control levels (3.6% ± 0.4%). However, two TNF-α concentrations inhibited 50 pmol/L CCK-stimulated secretion (13.8% ± 1.2% and 13.1% ± 0.9% for 10 and 100 ng/mL), which, when compared to CCK alone (18.5% ± 1.2%), represent a reduction of 25.4% and 29.2%, respectively.

TNF-α-induced actin cytoskeleton disorganization is increased during CCK stimulation

TNF-α has been shown to cause actin disorganization in isolated pancreatic acini, which is distinguished by increased filamentous actin along the basolateral membranes and its decrease in the apical area[21]. Here, we showed that both KRH vehicle-control and CCK-stimulated pancreatic acini exhibited the typical actin staining, intense in the apical area, and little and weak in the basolateral area of the acinar cell (Figure 1B and D). However, both 10 ng/mL TNF-α- and 10 ng/mL TNF-α

plus 50 pmol/L CCK-stimulated acini exhibited the previously described actin disorganization pattern, which was more pronounced in acini challenged with TNF-α plus CCK (Figure 1C and E). This disorganization was also observed when using 100 ng/mL, but not with 1 ng/mL TNF-α alone or together with CCK (data not shown). Figure 1F shows the quantification of actin distribution (0.13 ± 0.01, 0.11 ± 0.01, 0.33 ± 0.02 and 0.47 ± 0.03 basolateral/apical actin ratio for KRH, CCK, TNF-α and TNF-α + CCK, respectively).

TNF-α-induced activation of NF-κB is amplified by submaximal CCK

We then evaluated the activation of NF-κB (p65) and NF-κB (p50) in pancreatic acini. Previous reports established that pancreatic acinar cells respond to TNF-α by activating NF-κB in a PKCδ- and PKCε-dependent manner[22,23]. Although submaximal CCK stimulation of pancreatic acini induces the translocation and activation of PKCδ[29], this is not sufficient to activate NF-κB[29,30]. Here, we used the minimal TNF-α concentration (10 ng/mL) that we determined inhibited CCK-stimulated amylase secretion and induced actin disorganization (Figure 1A-F). Figure 2A and B show that CCK alone did not change the activity of NF-κB subunits over basal vehicle-control levels (1.03 ± 0.06 and 1.02 ± 0.05 fold of control for p65 and p50, respectively). However, TNF-α-induced an increase in the activity of both NF-κB subunits (1.52 ± 0.13 and 1.44 ± 0.11 fold of control for p65 and p50, respectively) that was amplified by CCK stimulation (3.28 ± 0.24 and 2.89 ± 0.20 fold of control for p65 and p50, respectively). Furthermore, we found that both TNF-α and TNF-α plus CCK-induced increases in the activities of NF-κB subunits were inhibited, not only by the non-specific PKC inhibitor calphostin C (iPKC∑) (p65, 1.04 ± 0.09 and 1.12 ± 0.11 fold of control for TNF-α and TNF-α + CCK, respectively; p50, 0.98 ± 0.04 and 1.14 ± 0.11 fold of control for TNF-α and TNF-α + CCK, respectively), but by the specific PKCδ translocation inhibitor δV1-1 (iPKCδ) (p65, 0.93 ± 0.07 and 1.05 ± 0.09 fold of control for TNF-α and TNF-α + CCK, respectively; p50, 1.12 ± 0.06 and 1.04 ± 0.11 fold of control for TNF-α and TNF-α + CCK, respectively), and by the specific PKCε translocation inhibitor εV1-2 (iPKCε) (p65, 1.0 ± 0.06 and 1.12 ± 0.10 fold of control for TNF-α and TNF-α + CCK, respectively; p50, 1.00 ± 0.06 and 1.12 ± 0.10 fold of control for TNF-α and TNF-α + CCK, respectively). Neither the specific PKCα inhibitor Gö6976 (iPKCα) nor the PKCζ myristoylated pseudosubstrate inhibitor (iPKCζ) modified the changes in the activities of the NF-κB subunits induced by TNF-α or TNF-α plus CCK.

TNF-α potentiates submaximal CCK-induced trypsin activity

We proceeded to analyze trypsin activity in pancreatic acini. Figure 2C shows that submaximal CCK alone only moderately increased trypsin activity (1.39 ± 0.12 fold of control), as was previously reported for submaximal cerulein[24,31,32]. While basal trypsin activity was not modified by TNF-α alone (1.09 ± 0.04 fold of control), its presence potentiated CCK-induced trypsin activity (2.63 ± 0.24 fold of control). Given that PKCδ and PKCε also modulate trypsin activity in pancreatic acinar cells[24], we further assessed trypsin activity in the presence of PKC inhibitors. We found that both CCK and TNF-α plus CCK-induced increases in trypsin activity were inhibited, not only by iPKC∑ (1.10 ± 0.09 and 1.61 ± 0.14 fold of control for CCK and TNF-α + CCK, respectively), but also by iPKCδ (1.12 ± 0.08 and 1.75 ± 0.15 fold of control for CCK and TNF-α + CCK, respectively). By contrast, iPKCε only inhibited TNF-α plus CCK-induced trypsin activity enhancement (1.38 ± 0.14 and 1.71 ± 0.12 fold of control for CCK and TNF-α + CCK, respectively). Furthermore, neither iPKCα nor iPKCζ modified the increase in trypsin activity induced by CCK or TNF-α plus CCK.

PKCδ and PKCε are translocated and activated by TNF-α plus submaximal CCK

To validate the results obtained with the specific PKC inhibitors, we examined the expression of PKCδ and PKCε in subcellular fractions from isolated pancreatic acini, as well as the respective kinase activity. Previous studies have shown that while PKCδ is translocated from cytosol to membranes and activated by both TNF-α and submaximal CCK, PKCε is only translocated and activated by TNF-α[23,29]. Here, we found similar results (1.35 ± 0.15 and 1.59 ± 0.14 fold of control for TNF-α and CCK, respectively, for PKCδ activity; 1.29 ± 0.09 and 1.02 ± 0.06 fold of control for TNF-α and CCK, respectively, for PKCε activity, Figure 2D-F). Furthermore, we showed that TNF-α plus submaximal CCK also translocated and activated both PKCδ and PKCε, significantly increasing the translocation and activation of PKCδ compared to either TNF-α or CCK alone (1.88 ± 0.12 and 1.41 ± 0.12 fold of control for TNF-α + CCK for PKCδ and PKCε, respectively, Figure 2D-F).

TNF-α does not perturb intracellular calcium oscillations induced by submaximal CCK

Both trypsinogen and NF-κB activation in pancreatic acini require not only PKC activity, but also calcium (Ca^{2+})[24,23,31,33]. To test the possibility that TNF-α could influence CCK activation of trypsinogen and/or NF-κB by directly affecting the Ca^{2+} signal, we studied the effects of TNF-α (10 ng/mL) on changes to the cytosolic free calcium concentration ($[Ca^{2+}]_i$) induced by CCK (50 pmol/L) in pancreatic acini. At submaximal concentrations, CCK induced oscillatory changes in $[Ca^{2+}]_i$ with a typical pattern consisting of base-line spikes[34]. Our results indicate that TNF-α did not affect the basal $[Ca^{2+}]i$ level in pancreatic acini, nor had any significant effects on the CCK-induced $[Ca^{2+}]i$ oscillations (data not shown).

TNF-α pus submaximal CCK induces necrosis in pancreatic acini

The process of necrosis produces damaged plasma mem-

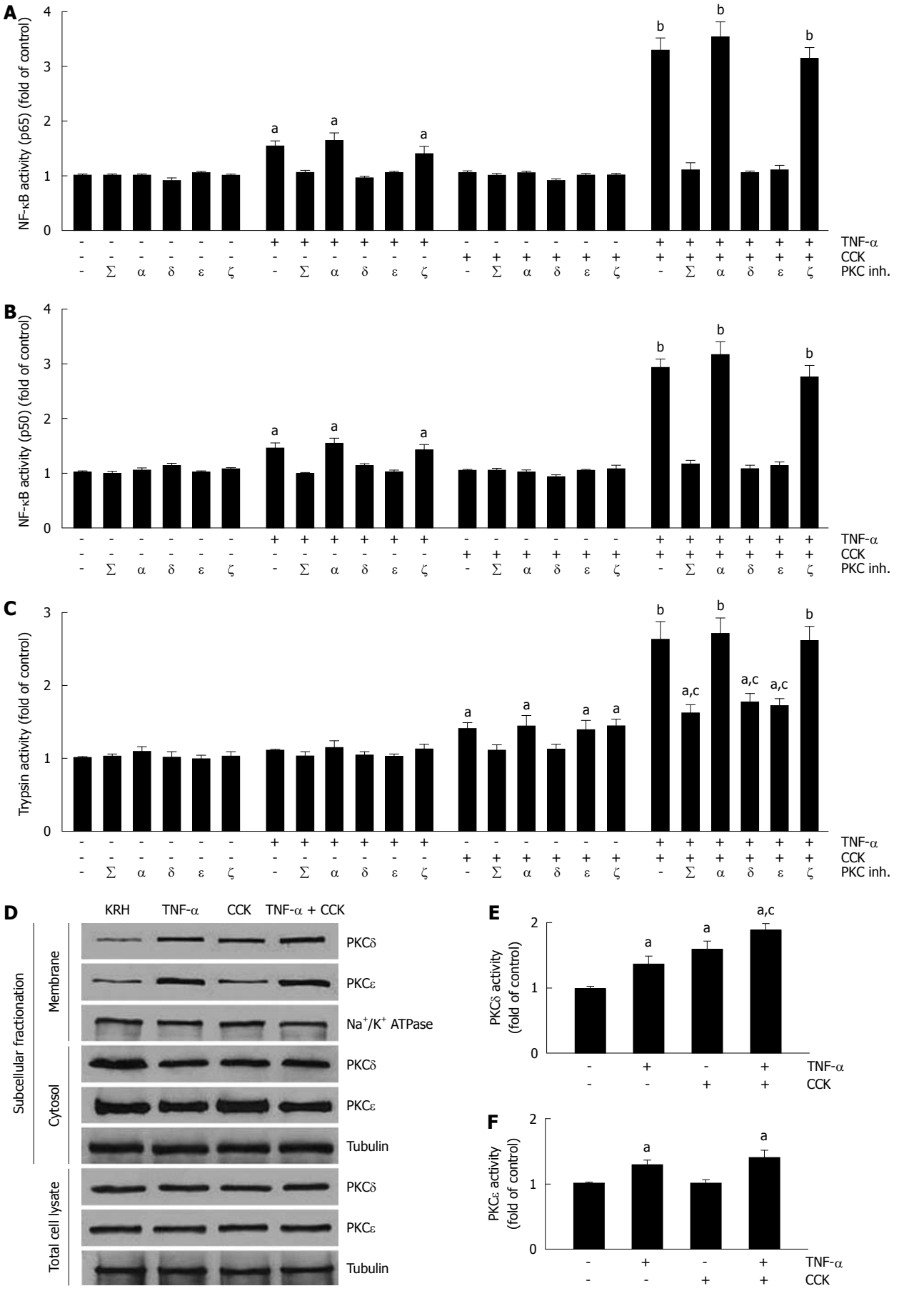

Figure 2 Mutual modulation of tumor necrosis factor-α-induced activation of nuclear factor-κB and cholecystokinin-induced trypsin activity by submaximal cholecystokinin and tumor necrosis factor-α, respectively, involves specific protein kinase C isoforms δ and ε. A-C: Isolated pancreatic acini were incubated in

Krebs-Ringer-HEPES (KRH) (vehicle-control, 3 h), or in KRH (2.5 h) followed by Calphostin C (iPKCΣ, 500 nmol/L, 30 min) or Gö6976 (iPKCα, 200 nmol/L, 30 min), or with the PKCδ translocation inhibitor δV1-1 (iPKCδ, 3 h, 10 μmol/L) or the PKCε translocation inhibitor εV1-2 (iPKCε, 3 h, 10 μmol/L) or the PKCζ myristoylated pseudosubstrate inhibitor (iPKCζ, 3 h, 10 μmol/L). The acini were then stimulated with either 10 ng/mL tumor necrosis factor-α (TNF-α) for 2 h, or 50 pmol/L cholecystokinin (CCK) for 1 h, or 10 ng/mL TNF-α for 1 h followed by 10 ng/mL TNF-α plus 50 pmol/L CCK for 1 h, or KRH for 2 h. Nuclear factor-κB (NF-κB) (p65) and NF-κB (p50) in nuclear extracts (A and B) were measured by enzyme-linked immunosorbent assay (ELISA) using the respective assay kit. Trypsin activity (C) was measured as described in Materials and Methods. A-C: Results are expressed as fold of control and correspond to the mean ± SE from four independent experiments, with samples performed in triplicate. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* KRH without inhibitors; $^{c}P < 0.05$ *vs* TNF-α plus CCK without inhibitors; D-F: Dispersed acini were pre-incubated in KRH for 1 h and then stimulated with either 10 ng/mL TNF-α for 1.5 h, or 50 pmol/L CCK for 30 min, or 10 ng/mL TNF-α for 1 h followed by 10 ng/mL TNF-α plus 50 pmol/L CCK for 30 min, or KRH (vehicle-control) for 1.5 h; D: Subcellular fractionation showing PKCδ and ε translocation from the cytosol to the membrane upon TNF-α and/or CCK stimulation. After stimulation, acini were fractionated into membrane and cytosol fractions, and total cell lysates. 10 μg of protein of each fraction was separated on SDS-PAGE and immunoblotted with the antibodies to the indicated proteins. These blots are representative of three independent experiments; E, F: PKCδ and ε activity assays. Each well was loaded with 10 μg of protein. PKCδ and ε activities were measured by ELISA using the respective assay kit. Results are expressed as fold of control and correspond to the mean ± SE from four independent experiments, with samples performed in triplicate. $^{a}P < 0.05$ *vs* vehicle-control; $^{c}P < 0.05$ *vs* either TNF-α or CCK.

branes, releasing LDH into the extracellular medium. Thus, to evaluate necrosis in the present study, we measured LDH release from pancreatic acini. Figure 3A shows that unstimulated acini exhibited a 6.1% ± 0.5% LDH release, which remained relatively unchanged when acini were stimulated with TNF-α or CCK alone (6.4% ± 0.7% and 6.8% ± 0.8%, respectively). However, acini stimulated with TNF-α plus CCK showed a significant increase of 83.6% for LDH release (11.2% ± 1.1%).

TNF-α plus submaximal CCK potentiates TNF-α-induced caspases' activity

Subsequently, we assessed the activity of caspases in pancreatic acini. Figure 3B shows that TNF-α does augment caspases' activity (1.22 ± 0.05, 1.25 ± 0.11, 1.24 ± 0.09 and 1.21 ± 0.08 fold of control for caspases 2, 3, 8, and 9, respectively). Although CCK alone did not change the activity of these pro-apoptotic proteins (1.02 ± 0.06, 1.03 ± 0.08, 1.04 ± 0.11 and 1.02 ± 0.06 fold of control for caspases 2, 3, 8, and 9, respectively), its combination with TNF-α further increased the activation achieved by TNF-α alone (1.93 ± 0.14, 2.14 ± 0.23, 1.87 ± 0.16 and 1.82 ± 0.19 fold of control for caspases 2, 3, 8, and 9, respectively).

TNF-α plus submaximal CCK depletes ATP and increases the ADP/ATP ratio in pancreatic acini

ATP depletion associated with the percentage of ATP loss and changes in the ADP/ATP ratio allows discrimination of apoptosis from necrosis. Thus, we analyzed ATP and ADP levels in pancreatic acini. Figure 3C shows that neither TNF-α nor CCK alone changed ATP levels compared to basal vehicle-control (10.8 ± 0.7, 11.6 ± 0.7, and 12.5 ± 0.2 μmol/mg protein for TNF-α, CCK and vehicle-control, respectively). However, TNF-α plus CCK significantly decreased ATP levels (4.3 ± 0.6 μmol/mg protein). As shown in Figure 3D, only TNF-α plus CCK significantly increased ATP loss (65.6% ± 4.8% of control), while TNF-α or CCK alone did not (13.6% ± 6.4% and 7.9% ± 5.6% of control, respectively). Figure 3E shows that only TNF-α plus CCK increased the ADP/ATP ratio over basal control (0.85 ± 0.12 *vs* 0.43 ± 0.01, respectively), while TNF-α or CCK alone did not (0.49 ± 0.04 and 0.46 ± 0.03, respectively).

Pancreatic acinar cells secrete TNF-α in response to different stress stimuli

It is well established that pancreatic acinar cells produce TNF-α[22]. Secretion of TNF-α in response to a stressor was previously demonstrated in rat pancreatic acinar cells by their exposure to phorbol-12-myristate-13-acetate (PMA)-primed neutrophils[19,20]. Here, we evaluated the response of pancreatic acini to hypoxia/reoxygenation (H/R) and hydrogen peroxide (H_2O_2) treatments. Figure 4A and B show that both stress stimuli increased TNF-α levels (3.2 ± 0.3 and 7.0 ± 0.6 fold of control for H/R and H_2O_2, respectively). Incubation of acini with the specific ROS inhibitor NAC reduced TNF-α levels (1.7 ± 0.2 and 3.8 ± 0.4 fold of control for H/R and H_2O_2, representing a reduction of 46.9% and 45.7%, respectively), indicating that ROS mediated these stress-induced TNF-α secretions.

Chronic stress plus submaximal cerulein stimulation induces mild to moderate pancreatitis

We then evaluated chronic stress plus submaximal cerulein pancreatitis in a rat model, together with an anti-TNF-α treatment as a putative therapy. First, we examined relevant serum and histological parameters of pancreatitis. Table 1 shows that Stress (+) treatment alone did not affect serum amylase and lipase levels, which were only slightly increased in the Stress (-) plus Cer group. However, this increase did not correlate with pancreatitis, which was confirmed by the completely normal pancreatic histology (Figure 5 and Table 2). In contrast, the Stress (+) plus Cer group exhibited a massive increase in serum amylase (8.4 ×) and lipase levels (85.4 ×) (Table 1) with corresponding histological changes of mild (cytoplasmic vacuoles) to moderate pancreatitis (hemorrhage, leukocyte infiltration, and necrosis) (Figure 5 and Table 2). Of particular relevance for our model, serum TNF-α was not detected in any of the Stress (-) groups (Table 1). Importantly, while TNF-α levels were detected in vehicle-control Stress (+) group, they further increased (3.5 ×) in the Stress (+) plus Cer group, which also demonstrated a marked augmentation (6.5 ×) in serum levels of LDH (Table 1). Of note, relevant serum parameters, as well as the histopathological changes found in the Stress (+) plus Cer group, were all diminished by the anti-TNF-α treatment (Figure 5, Tables 1 and 2).

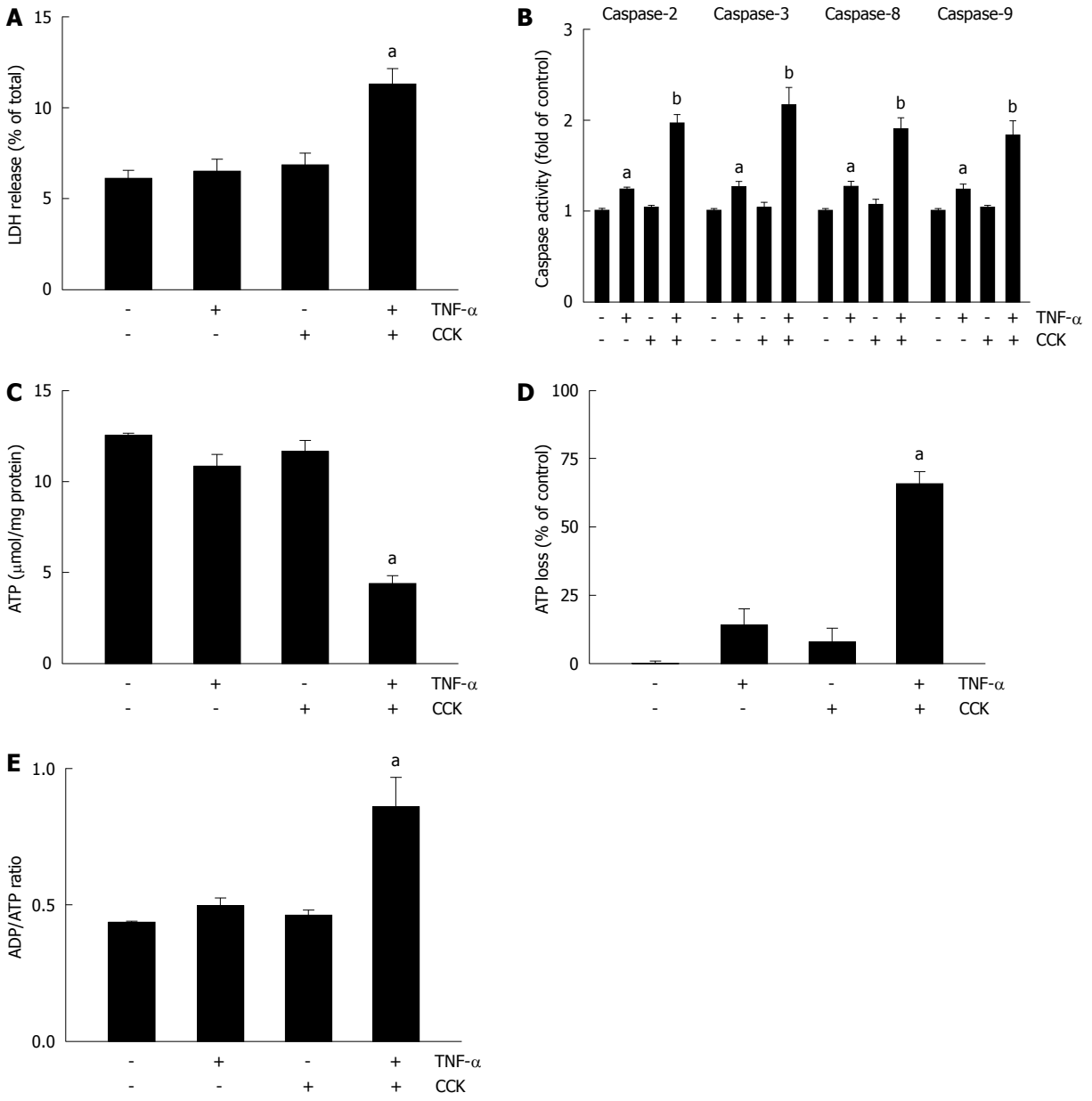

Figure 3 Tumor necrosis factor-α plus cholecystokinin induce apoptosis and necrosis in pancreatic acini. A-E: Isolated pancreatic acini were stimulated with either 10 ng/mL tumor necrosis factor-α (TNF-α) for 2 h, or 50 pmol/L cholecystokinin (CCK) for 1 h, or 10 ng/mL TNF-α for 1 h followed by 10 ng/mL TNF-α plus 50 pmol/L CCK for 1 h, or vehicle-control for 2 h. A: Lactate dehydrogenase (LDH) was measured by the DGKC optimized kinetic method using the respective assay kit. Results are expressed as a percentage of total cellular LDH determined by permeabilizing cells with Triton X-100, and correspond to the mean ± SE from four independent experiments, with samples performed in triplicate. $^{a}P < 0.05$ *vs* vehicle-control; B: Caspases 2, 3, 8, and 9 activity was measured by a colorimetric/fluorometric method using the respective assay kits. Results are expressed as fold of control and correspond to the mean ± SE from four independent experiments, with samples performed in triplicate. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* vehicle-control; C-E: ATP and ADP levels were measured by a bioluminescent method using the respective assay kits. ATP loss was calculated as a percentage of the decrease in values corresponding to vehicle-control. Results correspond to the mean ± SE from four independent experiments, with samples performed in triplicate. $^{a}P < 0.05$ *vs* vehicle-control.

We then analyzed inflammatory parameters in pancreatic tissue. The results are shown in Table 3. As was observed in serum, pancreatic TNF-α was only detected in the Stress (+) groups, where the levels were further increased (3.4 ×) in the Cer subgroup, and were diminished by 63% by the anti-TNF-α treatment. Remarkably, HIF-1α showed a similar trend, being only detected in the Stress (+) groups, where the levels were further increased (4 ×) in the Cer subgroup, and diminished by 58% by the anti-TNF-α treatment. Other assessed parameters (active trypsin, NF-κB (p65) and (p50), MPO, LPO, and MDA) also exhibited increased values in the Stress (+) plus Cer group, which were reduced by the anti-TNF-α treatment.

We also evaluated inflammatory and general biochemical parameters in blood. As shown in Table 4, all the studied inflammatory parameters (leukocyte number, C-reactive protein, HSP-72, IL-6, IL-10, and MIP-1α) were increased in the Stress (+) plus Cer group, whose values correlated with mild to moderate pancreatitis. Im-

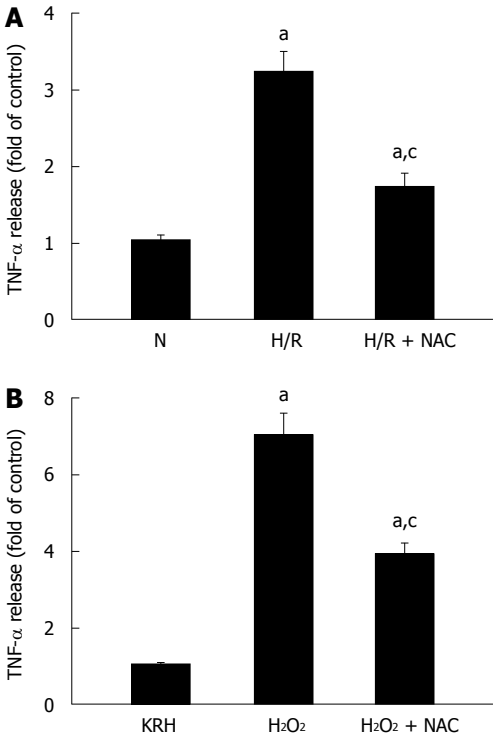

Figure 4 Pancreatic acinar cells secrete tumor necrosis factor-α in response to different stressful stimuli. A: Isolated pancreatic acini were cultured in normoxic (N) conditions for 3.5 h or under hypoxia followed by normoxia-reoxygenation (H/R) for 30 min and 3 h, respectively, in the absence or presence of N-acetyl-cysteine (NAC) (30 mmol/L); B: Isolated pancreatic acini were cultured in Krebs-Ringer-HEPES (KRH) (vehicle-control) or stimulated with H_2O_2 (50 μmol/L) for 3 h in the absence or presence of NAC (30 mmol/L); A and B: TNF-α released into the supernatant was determined by enzyme-linked immunosorbent assay using the respective assay kit. Results are expressed as fold of control and correspond to the mean ± SE from four independent experiments, with samples performed in triplicate. $^aP < 0.05$ *vs* normoxia (A) or to KRH (B); $^cP < 0.05$ *vs* H/R (A) or H_2O_2 (B).

portantly, these elevated values were all attenuated by the anti-TNF-α treatment. Furthermore, Table 5 shows that the studied biochemical parameters (glucose, urea, creatinine, calcium, proteins, AST, ALT, and hematocrit) also evidenced alterations compatible with mild to moderate pancreatitis in the Stress (+) plus Cer group, which were mitigated in the Stress (+) plus anti-TNF-α plus Cer group.

As performed for isolated pancreatic acini, we then investigated pancreatic caspases and energy metabolism in pancreatic tissue. Table 6 shows that caspases 2, 3, 8, and 9 were only augmented in the Stress (+) groups. These values were further increased (6-8 ×) in the Cer subgroup, and diminished by between 21%-37% by the anti-TNF-α treatment. Table 7 shows that the Stress (+) plus Cer group experienced a severe decrease in ATP content (> 70%) and a significant increase in the ADP/ATP ratio (3 ×). Both ATP loss and the ADP/ATP ratio were partially restored by the anti-TNF-α treatment.

Chronic stress plus submaximal cerulein stimulation induces pancreatitis-associated lung injury

A common, and often fatal, systemic complication of acute pancreatitis is acute lung injury, which frequently evolves to acute respiratory distress syndrome[35]. To evaluate this, we examined histological and inflammatory parameters in lung tissue. Figure 6 and Tables 8 and 9 show that histopathological parameters (edema, hemorrhage, and leukocyte infiltrate, Figure 6 and Table 8) as well as MPO, NF-κB (p65), and (p50) (Table 9) were uniformly normal in all Stress (-) groups. In contrast, the Stress (+) plus Cer group exhibited histopathological and inflammatory changes compatible with pancreatitis-associated lung injury (Figure 6, Tables 8 and 9). As was observed in pancreatic tissue, TNF-α levels were only detected in the Stress (+) groups, with increased values in the Cer subgroup (3 ×), which were attenuated by 57% by the anti-TNF-α treatment (Table 9).

Additionally, we measured caspases' activity and energy metabolism in lung tissue. The results are presented in Tables 10 and 11, respectively. As it was found for pancreatic tissue (Tables 6 and 7), caspases 2, 3, 8, and 9 were only augmented in the Stress (+) groups. These values were further increased (8-9 ×) in the Cer subgroup, and diminished between 30%-38% by the anti-TNF-α treatment (Table 10). Table 11 shows that the Stress (+) plus Cer group experienced a severe decrease in ATP content (> 65%) and a significant increase in the ADP/ATP ratio (4 ×). Here, the ATP loss and the ADP/ATP ratio were also partially restored by the anti-TNF-α treatment.

Table 1 Relevant parameters in serum (mean ± SE)

Group	Amylase (IU/L)	Lipase (IU/L)	TNF-α (pg/mL)	LDH (IU/L)
Stress (-)				
Veh	402 ± 23	12 ± 2	BD	299 ± 21
Cer	569 ± 31	44 ± 3	BD	358 ± 23
Anti-TNF-α + Cer	572 ± 43	42 ± 5	BD	327 ± 27
Stress (+)				
Veh	475 ± 21	21 ± 3	12 ± 2[a]	612 ± 41
Cer	3997 ± 226[a]	1793 ± 118[a]	42 ± 4[a]	3410 ± 218[a]
Anti-TNF-α + Cer	1895 ± 103[c]	714 ± 45[c]	16 ± 2[c]	1534 ± 121[c]

$^aP < 0.05$ *vs* control-vehicle Stress (-) group; $^cP < 0.05$ *vs* Stress (+) plus Cer group. BD: Below detection limit; LDH: Lactate dehydrogenase; TNF-α: Tumor necrosis factor-α.

Table 2 Histopathological score in pancreatic tissue (mean ± SE)

Group	Edema	Hemorrhage	Leukocyte infiltrate	Necrosis
Stress (-)				
Veh	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Cer	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Anti-TNF-α + Cer	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Stress (+)				
Veh	0.49 ± 0.06	0.00 ± 0.00	0.39 ± 0.05	0.00 ± 0.00
Cer	1.28 ± 0.11[a]	3.11 ± 0.17[a]	2.19 ± 0.10[a]	3.12 ± 0.15[a]
Anti-TNF-α + Cer	2.19 ± 0.17[c]	1.21 ± 0.17[c]	1.32 ± 0.10[c]	1.37 ± 0.19[c]

$^aP < 0.05$ *vs* control-vehicle Stress (-) group; $^cP < 0.05$ *vs* Stress (+) plus Cer group. TNF-α: Tumor necrosis factor-α.

Table 3 Inflammatory parameters in pancreatic tissue (mean ± SE)

Group	TNF-α (ng/mg DNA)	HIF-1α (ng/mg DNA)	Active trypsin (nmol/mg DNA)	NF-κB (p65) (μg/mg DNA)	NF-κB (p50) (μg/mg DNA)	MPO (U/mg DNA)	LPO (nmol/mg DNA)	MDA (μmol/mg DNA)
Stress (-)								
Veh	BD	BD	0.11 ± 0.02	43 ± 5	0.48 ± 0.04	0.00 ± 0.00	BD	0.71 ± 0.03
Cer	BD	BD	0.23 ± 0.04	46 ± 6	0.51 ± 0.05	0.00 ± 0.00	BD	0.89 ± 0.05
Anti-TNF-α + Cer	BD	BD	0.22 ± 0.05	44 ± 3	0.50 ± 0.03	0.00 ± 0.00	BD	0.84 ± 0.05
Stress (+)								
Veh	1.6 ± 0.2[a]	0.9 ± 0.1[a]	0.37 ± 0.06	72 ± 6	0.84 ± 0.07	1.41 ± 0.07	27 ± 2	2.05 ± 0.11
Cer	5.4 ± 0.3[a]	3.6 ± 0.3[a]	4.15 ± 0.32[a]	389 ± 25[a]	5.54 ± 0.35[a]	15.90 ± 0.66[a]	316 ± 20[a]	22.16 ± 0.69[a]
Anti-TNF-α + Cer	2.0 ± 0.2[c]	1.5 ± 0.3[c]	1.79 ± 0.21[c]	221 ± 18[c]	2.65 ± 0.24[c]	7.14 ± 0.38[c]	171 ± 14[c]	12.38 ± 0.47[c]

[a]$P < 0.05$ *vs* control-vehicle Stress (-) group; [c]$P < 0.05$ *vs* Stress (+) plus Cer group. BD: Below detection limit; TNF-α: Tumor necrosis factor-α; HIF-1α: Hypoxia inducible factor-1α; NF-κB: Nuclear factor-κB; MPO: Myeloperoxidase; LPO: Lipid hydroperoxide; MDA: Malondialdehyde.

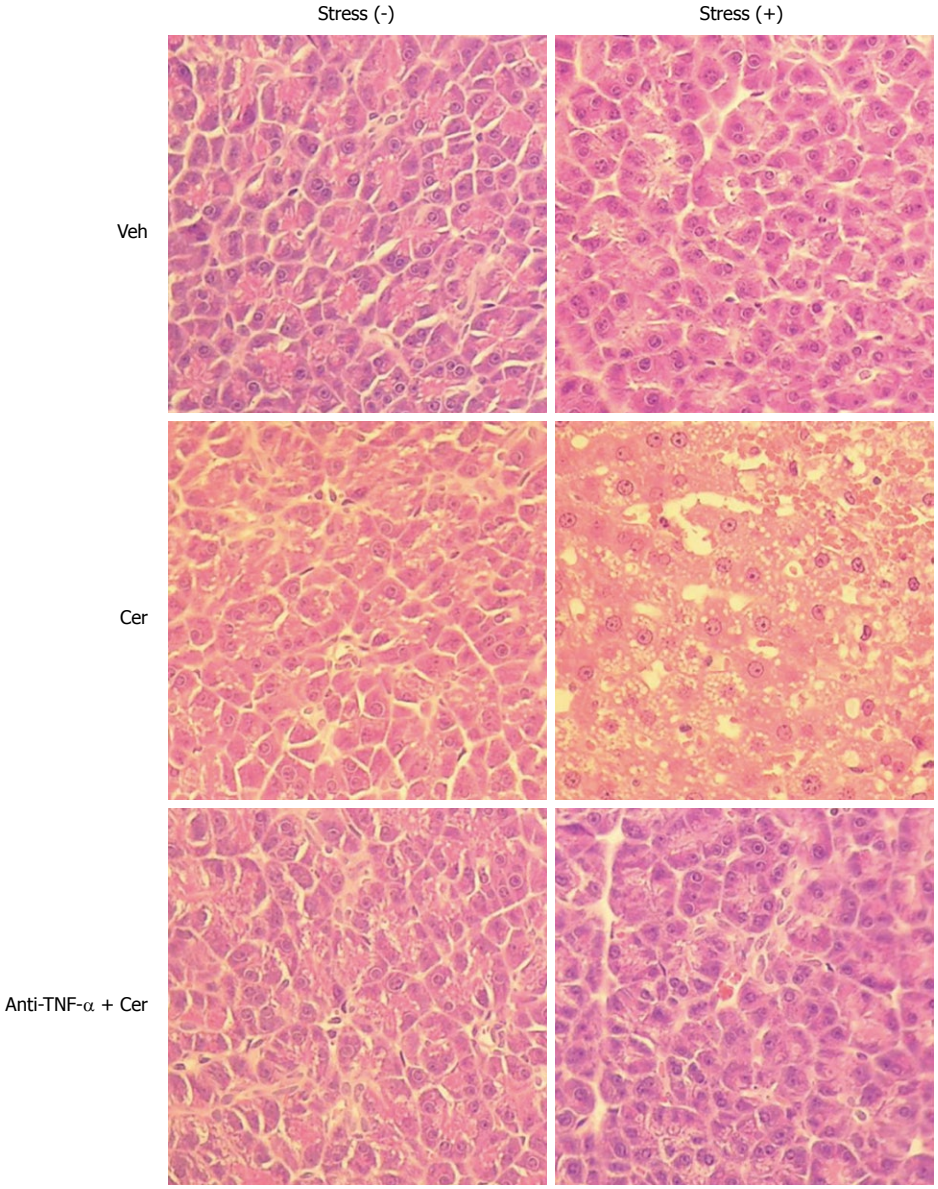

Figure 5 Chronic stress plus submaximal cerulein stimulation induces mild to moderate pancreatitis. Rats exposed to sessions of restraint (4 h every day for 21 d), Stress (+) groups, and rats in the control, Stress (-) groups, received daily intraperitoneal (ip) injections of either tumor necrosis factor-α (TNF-α)-neutralizing antibody (50 μg/kg) or control IgG. Rats were then treated with six hourly ip injections of either saline or cerulein (Cer, 0.2 μg/kg). Representative histology images of pancreatic tissue. HE, original magnification, 40 ×.

Table 4 Inflammatory parameters in blood (mean ± SE)

Group	Leukocytes (ng/mL)	CRP (mg/dL)	HSP-72 (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)	MIP-1α (pg/mL)
Stress (-)						
Veh	5900 ± 240	0.0 ± 0.0	558 ± 27	BD	BD	7 ± 1
Cer	6111 ± 248	0.0 ± 0.0	866 ± 32	BD	BD	9 ± 1
Anti-TNF-α + Cer	6005 ± 244	0.0 ± 0.0	817 ± 49	BD	BD	8 ± 1
Stress (+)						
Veh	7139 ± 250	1.5 ± 0.2[a]	899 ± 63	66 ± 6[a]	75 ± 5[a]	54 ± 4[a]
Cer	21222 ± 976[a]	24.9 ± 1.3[a]	2648 ± 159[a]	1115 ± 54[a]	1404 ± 148[a]	318 ± 23[a]
Anti-TNF-α + Cer	12733 ± 586[c]	12.4 ± 0.8[c]	1805 ± 112[c]	417 ± 38[c]	596 ± 68[c]	127 ± 11[b]

[a]$P < 0.05$ *vs* control-vehicle Stress (-) group; [c]$P < 0.05$ *vs* Stress (+) plus Cer group. CRP: C-reactive protein; HSP: Heat shock protein; IL: Interleukin; MIP-1α: Macrophage inflammatory protein-1α; TNF-α: Tumor necrosis factor-α; BD: Below detection limit.

Table 5 General biochemical assays in blood (mean ± SE)

Group	Glucose (mg/dL)	Urea (mg/dL)	Creatinine (mg/dL)	Calcium (mg/dL)	Proteins (g/dL)	AST (IU/L)	ALT (IU/L)	Hematocrit (%)
Stress (-)								
Veh	139 ± 13	39 ± 4	0.48 ± 0.03	13.02 ± 0.57	8.51 ± 0.39	192 ± 21	88 ± 7	40 ± 5
Cer	153 ± 14	55 ± 4	0.65 ± 0.09	11.08 ± 0.48	7.46 ± 0.28	298 ± 32	155 ± 14	42 ± 4
Anti-TNF-α + Cer	148 ± 12	52 ± 5	0.67 ± 0.08	10.91 ± 0.70	7.51 ± 0.43	293 ± 20	149 ± 9	41 ± 5
Stress (+)								
Veh	118 ± 11[a]	38 ± 5	0.50 ± 0.05	11.72 ± 0.61	8.48 ± 0.22	201 ± 26	95 ± 9	36 ± 5[a]
Cer	294 ± 26[a]	105 ± 9[a]	1.94 ± 0.50[a]	9.03 ± 0.33[a]	6.02 ± 0.19[a]	432 ± 35[a]	219 ± 19[a]	41 ± 3
Anti-TNF-α + Cer	189 ± 16[c]	73 ± 8[c]	1.14 ± 0.24[c]	9.63 ± 0.58	6.76 ± 0.35[c]	356 ± 29[c]	187 ± 19[c]	39 ± 4

[a]$P < 0.05$ *vs* control-vehicle Stress (-) group; [c]$P < 0.05$ *vs* Stress (+) plus Cer group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TNF-α: Tumor necrosis factor-α.

Table 6 Pancreatic caspases (mean ± SE)

Group	CASP-2	CASP-3	CASP-8	CASP-9
Stress (-)				
Veh	34 ± 6	25 ± 2	45 ± 5	40 ± 7
Cer	56 ± 12	63 ± 9	87 ± 11	82 ± 8
Anti-TNF-α + Cer	50 ± 7	39 ± 5	72 ± 7	68 ± 9
Stress (+)				
Veh	124 ± 15	118 ± 10	189 ± 11	158 ± 16
Cer	995 ± 88[a]	838 ± 74[a]	1123 ± 103[a]	1079 ± 70[a]
Anti-TNF-α + Cer	696 ± 47[c]	544 ± 58[c]	887 ± 65[c]	679 ± 48[c]

Pancreatic caspases are expressed as pmol/min per milligram protein. [a]$P < 0.05$ *vs* control-vehicle Stress (-) group; [c]$P < 0.05$ *vs* Stress (+) plus Cer group. TNF-α: Tumor necrosis factor-α; CASP: Caspase.

Table 7 Energy metabolism in pancreatic tissue (mean ± SE)

Group	ATP (μmol/mg protein)	ATP loss (%)	ADP/ATP ratio
Stress (-)			
Veh	9.1 ± 0.4	0.0 ± 4.4	0.32 ± 0.04
Cer	6.8 ± 0.5	25.3 ± 5.5	0.38 ± 0.05
Anti-TNF-α + Cer	6.9 ± 0.6	24.2 ± 6.6	0.36 ± 0.04
Stress (+)			
Veh	7.3 ± 0.5	19.8 ± 5.5	0.40 ± 0.06
Cer	2.6 ± 0.4[a]	71.4 ± 4.4[a]	0.94 ± 0.11[a]
Anti-TNF-α + Cer	5.3 ± 0.3[c]	41.8 ± 3.3[c]	0.63 ± 0.07[c]

[a]$P < 0.05$ *vs* control-vehicle Stress (-) group; [c]$P < 0.05$ *vs* Stress (+) plus Cer group. TNF-α: Tumor necrosis factor-α.

Chronic stress induces gastric lesions

In the general adaptation syndrome described by Selye, the first lesions to appear as a result of stress were found in the stomach. To corroborate that the protocol we have used in our model produces stress, we evaluated histopathological changes together with apoptotic/necrotic parameters in stomach tissue. Tables 12-14 show that our protocol of restraint was effective in generating stress in rats.

DISCUSSION

Although oxidative stress and inflammation each occur in the pancreas during the early stage of supramaximal cerulein-induced acute pancreatitis, oxidative stress or an inflammatory insult alone does not cause the characteristic changes of acute pancreatitis[36]. However, our present study demonstrates that chronic stress leaves the exocrine pancreas susceptible to pancreatitis by submaximal cerulein stimulation.

The main events occurring in the pancreatic acinar cell that initiate and propagate acute pancreatitis include inhibition of secretion, intracellular activation of proteases, and generation of inflammatory mediators[37]. These cellular events can be correlated with the acinar morphological changes (retention of enzyme content, formation of large vacuoles containing both digestive enzymes and lysosomal hydrolases, and necrosis), which are observed in the well-established *in vivo* experimental model of supraphysiological cerulein-induced pancreatitis[38], as well as in human acute pancreatitis[39].

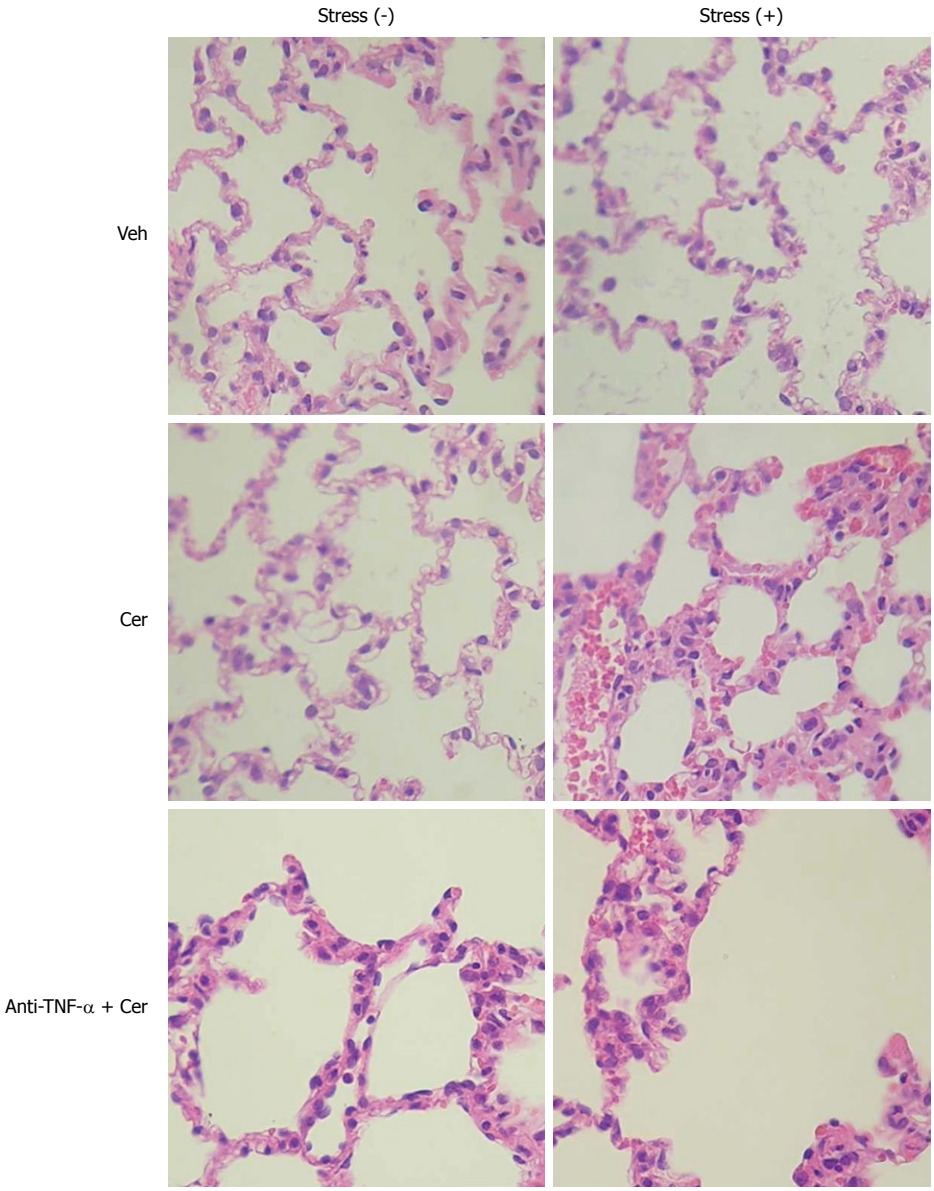

Figure 6 Chronic stress plus submaximal cerulein stimulation induces pancreatitis-associated lung injury. Rats exposed to sessions of restraint (4 h every day for 21 d), Stress (+) groups, and rats in the control, Stress (-) groups, received daily intraperitoneal (ip) injections of either tumor necrosis factor-α (TNF-α)-neutralizing antibody (50 μg/kg) or control IgG, rats were then treated with six hourly ip injections of either saline or cerulein (Cer, 0.2 μg/kg). Representative histology images of lung tissue. HE, original magnification, 40 ×.

Table 8 Histopathological score in lung tissue (mean ± SE)

Group	Edema	Hemorrhage	Leukocyte infiltrate
Stress (-)			
Veh	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Cer	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Anti-TNF-α + Cer	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Stress (+)			
Veh	0.36 ± 0.04	0.00 ± 0.00	0.30 ± 0.03
Cer	1.51 ± 0.11[a]	2.73 ± 0.58[a]	1.63 ± 0.54[a]
Anti-TNF-α + Cer	1.80 ± 0.21[c]	1.21 ± 0.08[c]	0.73 ± 0.05[c]

[a]$P < 0.05$ *vs* control-vehicle Stress (-) group; [c]$P < 0.05$ *vs* Stress (+) plus Cer group. TNF-α: Tumor necrosis factor-α.

Table 9 Inflammatory parameters in lung tissue (mean ± SE)

Group	TNF-α (ng/mg DNA)	MPO (U/mg DNA)	NF-κB (p65) (μg/mg DNA)	NF-κB (p50) (μg/mg DNA)
Stress (-)				
Veh	BD	0.00 ± 0.00	23 ± 2	0.27 ± 0.02
Cer	BD	0.00 ± 0.00	24 ± 2	0.30 ± 0.02
Anti-TNF-α + Cer	BD	0.00 ± 0.00	24 ± 3	0.29 ± 0.03
Stress (+)				
Veh	1.3 ± 0.1[a]	0.89 ± 0.12	43 ± 5	0.51 ± 0.03
Cer	4.2 ± 0.3[a]	12.10 ± 0.56[a]	251 ± 19[a]	2.92 ± 0.28[a]
Anti-TNF-α + Cer	1.8 ± 0.2[c]	5.04 ± 0.29[c]	107 ± 9[c]	1.13 ± 0.11[c]

[a]$P < 0.05$ *vs* control-vehicle Stress (-) group; [c]$P < 0.05$ *vs* Stress (+) plus Cer group. TNF-α: Tumor necrosis factor-α; MPO: Myeloperoxidase; NF-κB: Nuclear factor-κB.

Table 10 Lung caspases (mean ± SE)

Group	CASP-2	CASP-3	CASP-8	CASP-9
Stress (-)				
Veh	18 ± 3	20 ± 2	21 ± 5	22 ± 4
Cer	27 ± 3	29 ± 4	32 ± 5	29 ± 3
Anti-TNF-α + Cer	26 ± 2	24 ± 3	24 ± 4	22 ± 3
Stress (+)				
Veh	97 ± 11	103 ± 9	99 ± 8	104 ± 12
Cer	853 ± 81[a]	912 ± 101[a]	824 ± 77[a]	917 ± 85[a]
Anti-TNF-α + Cer	597 ± 48[c]	592 ± 55[c]	560 ± 45[c]	568 ± 47[c]

Lung caspases are expressed as pmol/min per milligram protein. [a]$P < 0.05$ *vs* control-vehicle Stress (-) group; [c]$P < 0.05$ *vs* Stress (+) plus Cer group. TNF-α: Tumor necrosis factor-α; CASP: Caspase.

Table 11 Energy metabolism in lung tissue (mean ± SE)

Group	ATP (μmol/mg protein)	ATP loss (%)	ADP/ATP ratio
Stress (-)			
Veh	10.3 ± 0.5	0.0 ± 4.9	0.20 ± 0.03
Cer	10.0 ± 0.3	2.9 ± 2.9	0.22 ± 0.02
Anti-TNF-α + Cer	10.1 ± 0.4	1.9 ± 3.8	0.21 ± 0.03
Stress (+)			
Veh	7.9 ± 0.7	25.5 ± 6.8	0.32 ± 0.05
Cer	3.6 ± 0.4[a]	66.0 ± 3.8[a]	0.72 ± 0.10[a]
Anti-TNF-α + Cer	6.7 ± 0.6[c]	36.8 ± 5.7[c]	0.49 ± 0.06[c]

[a]$P < 0.05$ *vs* control-vehicle Stress (-) group; [c]$P < 0.05$ *vs* Stress (+) plus Cer group. TNF-α: Tumor necrosis factor-α.

Table 12 Histopathological score in stomach tissue (mean ± SE)

Group	Edema	Hemorrhage	Leukocyte infiltrate	Necrosis
Stress (-)				
Veh	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Cer	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Anti-TNF-α + Cer	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Stress (+)				
Veh	3.25 ± 0.14[b]	1.55 ± 0.08[a]	1.26 ± 0.19[a]	0.95 ± 0.06[a]
Cer	1.17 ± 0.08[a]	3.23 ± 0.29[a]	2.77 ± 0.16[a]	3.48 ± 0.32[a]
Anti-TNF-α + Cer	1.96 ± 0.17[c]	2.23 ± 0.15[c]	1.63 ± 0.05[c]	1.57 ± 0.12[c]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* control-vehicle Stress (-) group; [c]$P < 0.05$ *vs* Stress (+) plus Cer group. TNF-α: Tumor necrosis factor-α.

Table 13 Stomach caspases (mean ± SE)

Group	CASP-2	CASP-3	CASP-8	CASP-9
Stress (-)				
Veh	37 ± 4	32 ± 3	48 ± 5	39 ± 5
Cer	40 ± 5	32 ± 5	51 ± 4	40 ± 3
Anti-TNF-α + Cer	33 ± 4	30 ± 3	47 ± 4	39 ± 4
Stress (+)				
Veh	1358 ± 132[a]	1515 ± 154[a]	1479 ± 136[a]	1425 ± 126[a]
Cer	798 ± 87[a]	824 ± 95[a]	868 ± 70[a]	832 ± 87[a]
Anti-TNF-α + Cer	1089 ± 98[c]	1203 ± 107[c]	1108 ± 89[c]	1144 ± 102[c]

Stomach caspases are expressed as pmol/min per milligram protein. [a]$P < 0.05$ *vs* control-vehicle Stress (-) group; [c]$P < 0.05$ *vs* Stress (+) plus Cer group. TNF-α: Tumor necrosis factor-α; CASP: Caspase.

Table 14 Energy metabolism in stomach tissue (mean ± SE)

Group	ATP (μmol/mg protein)	ATP loss (%)	ADP/ATP ratio
Stress (-)			
Veh	13.4 ± 0.9	0.0 ± 6.7	0.16 ± 0.02
Cer	12.9 ± 0.7	3.7 ± 5.2	0.18 ± 0.03
Anti-TNF-α + Cer	13.0 ± 0.8	3.0 ± 6.0	0.17 ± 0.02
Stress (+)			
Veh	5.5 ± 0.4[a]	58.9 ± 3.0[a]	0.67 ± 0.07[a]
Cer	2.3 ± 0.4[a]	82.8 ± 3.0[a]	1.14 ± 0.16[a]
Anti-TNF-α + Cer	6.2 ± 0.6[c]	53.7 ± 4.5[c]	0.58 ± 0.06[c]

[a]$P < 0.05$ *vs* control-vehicle Stress (-) group; [c]$P < 0.05$ *vs* Stress (+) plus Cer group. TNF-α: Tumor necrosis factor-α.

Here, we employed a new model of chronic stress plus submaximal cerulein stimulation to induce pancreatitis. Furthermore, we studied a new *in vitro* model of TNF-α plus submaximal CCK stimulation, and obtained results that not only reproduced the main pathological events experienced by the acinar cell, but also support the pancreatitis observed in animals exposed to the *in vivo* model.

First, we established that the pro-inflammatory cytokine TNF-α inhibits submaximal CCK-stimulated amylase secretion from pancreatic acini. While TNF-α alone does not stimulate amylase secretion in human pancreas[40] or in isolated rat pancreatic acini, it certainly alters the typical filamentous actin distribution[21]. A similar redistribution of actin from apical to basolateral membranes was observed in pancreatic acini supra-stimulated with CCK[41]. Disorganization of the actin cytoskeleton is associated with dysregulation of pancreatic enzyme secretion[42], which could explain our findings (Figure 1).

Although necessary, the inhibition of pancreatic enzyme secretion alone is not sufficient to induce pancreatitis[37]. Nonetheless, we also demonstrated that the combination of TNF-α and submaximal CCK pathologically activates NF-κB and trypsinogen in pancreatic acini. TNF-α has been shown to regulate the activity of distinct PKC isoforms in diverse cell types, including the pancreatic acinar cell[23,43,44]. PKC consists of a family of at least 12 isozymes differing in tissue distribution and activation requirements. There are three subclasses: classical PKC isozymes (-α, -β1, -β2, and -γ), which require calcium and are activated by diacylglycerol and phorbol ester; the novel PKC isozymes (-δ, -ε, -η, and -θ), which are activated by diacylglycerol and phorbol ester independently of calcium; and the atypical PKC isozymes (-λ, -ι, and -ζ), which are calcium independent and not responsive to phorbol ester. Rat pancreatic acini express the α, δ, ε, and ζ PKC isozymes[45]. Changes in PKC activity are associated with inflammation in a variety of tissues, including skin, kidney, intestine, and pancreas[46-49]. Specifically, PKC-δ and PKC-ε regulate the signal transduction pathways implicated in the pathophysiological activation of NF-κB and trypsinogen in pancreatic acini[23,24]. TNF-α activates both PKC-δ and PKC-ε in pancreatic acini[23], and our study shows

that in turn, these convert physiological CCK concentrations into physiopathogenic concentrations (Figure 2). Different studies have consistently shown that modulation of PKC activity sensitizes acinar cells to physiological CCK and cerulein treatments, resulting in harmful levels of NF-κB and trypsin activity, respectively[29,24]. In agreement with TNF-α-regulated functions[11] and the above discussed results, we found that TNF-α plus submaximal CCK induced both apoptosis and necrosis in acinar cells, as revealed by the increased caspases' activity, increased LDH release, ATP loss, and changes in the ADP/ATP ratio (Figure 3).

Secretion of TNF-α by several stress stimuli has been demonstrated *in vitro* in many cell types, including pancreatic acinar cells[13-20], and *in vivo* in different tissues[10,12,50,51]. We have shown that *in vitro* hypoxia-reoxygenation conditions also induce TNF-α secretion by acinar cells (Figure 4). These conditions are concomitant with ischemia-reperfusion processes, which can be the result of microcirculatory disturbances generated by stress[52]. Indeed, local pancreatic blood flow is reduced by stress[53]. Hence, alternate vasoconstriction and vasodilatation leading to tissue ischemia and reperfusion could reflect the putative local origin of chronic stress-derived TNF-α found in the rat exocrine pancreas, which is supported by the increased levels of HIF-1α observed in the Stress (+) groups (Figure 5, Tables 1-3). HIF-1α is a transcription factor induced by hypoxic conditions and is involved in different inflammatory processes, such as dermatitis, rheumatoid arthritis[54], and also pancreatitis[55].

Our model of chronic stress plus submaximal doses of cerulein produced the hallmark features of pancreatitis (Figure 5, Tables 1-5), and also of its most frequent complication, pancreatitis-associated lung injury (Figure 6, Tables 8 and 9), which characterizes the severity of this disease[34]. Importantly, treatment with a TNF-α antibody greatly protected rats from chronic stress-related pancreatitis (Figures 5 and 6, Tables 1-5, 8 and 9). These results strongly suggest a causative role for TNF-α in the development of pancreatitis induced in chronically stressed animals.

Although anti-TNF-α therapy decreased pancreatitis severity, animals still developed a very-mild form of this disease. The limited protection achieved *in vivo* was probably due to a multiplicity of factors involved in the development of pancreatitis, some of which seem to act independent of TNF-α. Further work will be required to identify the additional molecules and signaling pathways involved in chronic stress as a contributing factor to pancreatitis. Nevertheless, this initial work demonstrates a novel susceptibility mechanism by which chronic stress could predispose the exocrine pancreas to pancreatitis triggered by physiological levels of cerulein stimulation.

COMMENTS

Background

Pancreatitis is an inflammatory disorder of the pancreas characterized by premature activation of pancreatic enzymes leading to pancreatic autodigestion, persistent inflammation, edema, and possible pancreatic necrosis. A wide variety of factors, including gallstones, alcoholism, and a complication of endoscopic retrograde cholangiopancreatography, are thought to contribute to the pathogenesis of this disease.

Research frontiers

Initial inflammation of the pancreas may progress to involve other organs such as the kidney, lungs, and liver. Inflammatory mediators induce complications that can range from local to systemic, leading to a severe form with high morbidity/mortality. Thus, it is critical to identify the precipitating factors of pancreatitis, because treatment will diverge significantly depending on the etiology.

Innovations and breakthroughs

Chronic stress has received little attention as a factor contributing to pancreatitis. Using a new model combining chronic restraint and physiological stimulation of the exocrine pancreas, the authors have established that chronic stress might sensitize the pancreas to pancreatitis. Furthermore, they have begun to elucidate the molecules responsible for the initial events.

Applications

The protective effect shown by anti-tumor necrosis factor-α therapy in this experimental model of pancreatitis indicates a potential clinical application for this antibody in treating this disease. However, the results also indicate that other molecules might be involved in this pathology. This suggests that better results could be obtained by combined therapies, but this requires further experiments to identify additional key contributing molecules.

Peer review

The authors present a study that demonstrates that chronic stress enhances the susceptibility for pancreatic damage produced by physiological cerulein stimulation. It is thought that the authors have produced a good piece of work.

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ORIGINAL ARTICLE

Screening compounds against HCV based on MAVS/IFN-β pathway in a replicon model

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Abstract

AIM: To develop a sensitive assay for screening compounds against hepatitis C virus (HCV).

METHODS: The proteolytic cleavage of NS3/4A on enhanced yellow fluorescent protein (eYFP)-mitochondrial antiviral signaling protein (MAVS) was examined by reporter enzyme secreted placental alkaline phosphatase (SEAP), which enabled us to perform ongoing monitoring of anti-HCV drugs through repeated chemiluminescence. Subcellular localization of eYFP-MAVS was assessed by fluorescence microscopy. Cellular localization and protein levels were examined by Western blotting.

RESULTS: HCV NS3/4A protease cleaved eYFP-MAVS from mitochondria to block the activation of interferon (IFN)-β promoter, thus resulting in downregulation of SEAP activity. The decrease in SEAP activity was proportional to the dose of active NS3/4A protease. Also this reporter assay was used to detect anti-HCV activity of IFN-α and cyclosporine A.

CONCLUSION: Our data show that this reporter system is a sensitive and quantitative reporter of anti-HCV inhibitors. This system will constitute a new tool to allow the efficient screening of HCV inhibitors.

Key words: Mitochondrial antiviral signaling protein; Hepatitis C virus; Interferon-β; Drug screening

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INTRODUCTION

Hepatitis C virus (HCV) infection represents a major public health threat because of the chronic nature of the infection, its high prevalence and the significant morbidity of the resultant disease[1-3]. There is no vaccine, and drug treatment is costly and has poor efficacy[4,5]. New treatment regimens that are more efficacious and better tolerated by all patients are needed. Many new antiviral compounds have been synthesized; several of which have shown promising results in early clinical trials[6,7]. However,

development of new drugs is hampered by the lack of appropriate model systems. Therefore, there is an obvious and urgent need to develop a more effective and sensitive assay to facilitate the search for improved antivirals.

HCV is an enveloped virus that contains a single-stranded, positive-sense RNA genome of approximate 9.6 kb. The HCV genome encodes a large open reading frame that is flanked by structured 5' and 3' non-translated regions. There are at least 10 mature proteins that are contained in the polyprotein in the order: NH_2-core/E1/E2/p7/NS2/NS3/NS4A/NS4B/NS5A/NS5B-COOH, which is post-translationally processed. Processing of the nonstructural part of the polyprotein is mediated by the nonstructural NS3/4A protease and helicase[8-10]. Also, NS3/4A blocks the cellular interferon (IFN) response to double-stranded RNA by proteolytic cleavage of mitochondrial antiviral signaling protein (MAVS; also known as IPS-1, VISA, and CARDIF) or of the adaptor molecule Toll/interleukin-1 receptor domain-containing adaptor inducing IFN-β (TRIF)[11-16].

The ability of NS3/4A protease to control the antiviral host response to HCV facilitated our study to develop a new assay for drug screening. Here, we extended the utility of enhanced yellow fluorescent protein (eYFP)-MAVS/IFN-β-secreted placental alkaline phosphatase (SEAP) signaling pathway for anti-HCV drug discovery. Expression of NS3/4A protease in replicon cells disrupted the eYFP-MAVS/IFN-β-SEAP signaling pathway by proteolytic cleavage of eYFP-MAVS, which resulted in the loss of mitochondrial localization and abrogation of the eYFP-MAVS/IFN-β-SEAP signaling pathway. The decrease in SEAP activity was proportional to the dose of active NS3/4A protease. This reporter assay also was used to detect the activity of IFN-α and cyclosporine A (CsA). Our data show that this reporter system is a sensitive and quantitative reporter assay for anti-HCV inhibitors. This system will constitute a new tool to allow the efficient screening of HCV inhibitors.

MATERIALS AND METHODS

Plasmids

Plasmids eYFP-MAVS and pCon1-FL were kindly provided by Martin Baril and Dr. Charles M. Rice of Rockefeller University, respectively. Reporter plasmid of IFN-β-SEAP controlled by the promoter region of IFN-β (from -281 to 20) was constructed based on pTAL-SEAP (Clontech, Mountain View, CA, USA). The NS3/4A sequence derived from HCV type 1b (pCon1-FL) was cloned into pCI Mammalian Expression Vector using *Eco*RI and *Sal*I sites.

Cell culture and transfection

Huh-7.5 and HCV replicon cells that contained full-length HCV sequences of genotype 1b were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Biochrom AG, Berlin, Germany) and penicillin (100 μg/mL)/streptomycin (100 μg/mL). In 24-well plates, cells were seeded at a density of 6×10^4 cells per well in 500 μL DMEM/10% FBS. After incubation at 37℃ overnight, or until cells were approximately 70% confluent, cells were transfected with various plasmid constructs using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA). As an internal control for transfection efficiency, 0.02 μg Renilla luciferase plasmid, pRL-TK (Promega, Madison, WI, USA) was co-transfected with 1 μg of each expression vector in each experiment.

Treatment of cells with HCV inhibitors

Huh-7.5 and HCV replicon cells were seeded in 24-well plates at a density of 2×10^4 cells per well. After incubation at 37℃ overnight, cells were treated with various concentrations of IFN-α (Schering-Plough, Kenilworth, NJ, USA) or CsA (Sigma, St Louis, MO, USA). Two or three days later, cells were transfected with eYFP-MAVS and IFN-β-SEAP with the same concentration of drugs, and cells were incubated for one or two more days. Cell culture was collected and SEAP activity was measured by chemiluminescence. NS3 expression level in replicon cells was determined by Western blotting.

SEAP activity

SEAP activity was measured using the Phospha-Light™ assay kit (Tropix, Foster City, CA, USA) according to the manufacturer's instructions.

Western blotting

Protein concentration was estimated by BCA™ Protein Assay Kit from Pierce (Rockford, IL, USA). Cell lysates were separated by SDS-PAGE, and protein expression was detected by Western blotting analysis using antibodies. eYFP was detected using a rabbit polyclonal antibody (Proteintech, Chicago, IL, USA), actin was detected using a rabbit polyclonal antibody (Sigma), NS3 was detected using a goat polyclonal antibody (LifeSpan BioSciences, Seattle, WA, USA), with horseradish peroxidase (HRP)-conjugated secondary antibody followed by detection by chemiluminescent HRP substrate (Millipore, Billerica, MA, USA).

Statistical analysis

All results were expressed as the mean ± SD. Statistical comparisons between two groups were made using Student's *t* test after analysis of variance. The level of significance was set at $\alpha = 0.05$.

RESULTS

Activation of the IFN-β promoter by eYFP-MAVS

We assessed signaling activity of eYFP-MAVS in Huh7.5 cells by co-transfection alongside IFN-β-SEAP. As expected, eYFP-MAVS induced the activation of the IFN-β promoter. As shown in Figure 1, at 48 h post-transfection, eYFP-MAVS gave rise to an approximately 700-fold increase in SEAP activity. Subcellular localization of eYFP-MAVS was also assessed by fluorescence microscopy, with cells expressing eYFP-MAVS proteins. Prior to visualization, mitochondria and nuclei were labeled with

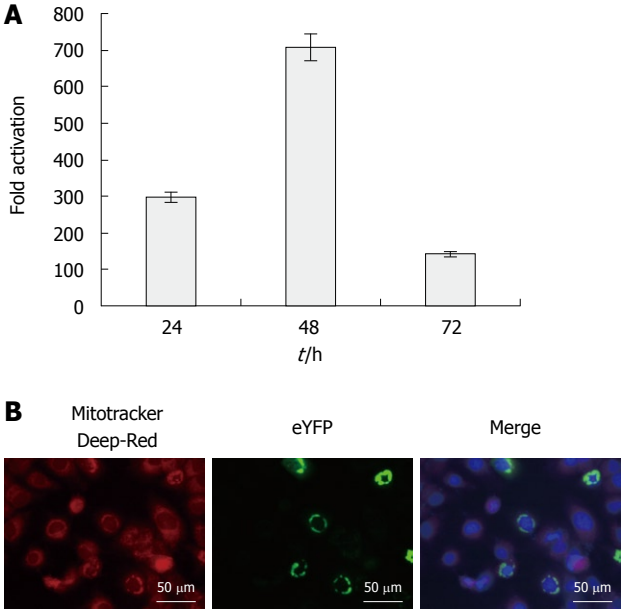

Figure 1 Activation of the interferon-β promoter by enhanced yellow fluorescent protein-mitochondrial antiviral signaling protein and subcellular localization of enhanced yellow fluorescent protein-mitochondrial antiviral signaling protein. A: Activation of the interferon (IFN)-β promoter by enhanced yellow fluorescent protein (eYFP)-mitochondrial antiviral signaling protein (MAVS). Expression vector of eYFP-MAVS was co-transfected with IFN-β-secreted placental alkaline phosphatase (SEAP) in Huh7.5 cells. pRL-TK was co-transfected to normalize transfection efficiency. SEAP activity in cell culture was measured at 24, 48 and 72 h post-transfection. Results are expressed as activation levels of the promoter compared to those in cells transfected with an empty expression vector. The error bars represent the SDs from the mean values obtained from three independent experiments performed in duplicate; B: Fluorescence microscopy of Huh7.5 cells transfected with eYFP-MAVS at 48 h post-transfection. Mitochondria were stained with Mitotracker deep red (red) and nuclei were labeled with 4',6-Diamidino-2-phenylindole (blue). Yellow labeling in the merged image indicates co-localization of eYFP-MAVS with mitochondria.

Mitotracker deep red and 4',6-Diamidino-2-phenylindole (DAPI), respectively. Figure 1 shows eYFP-MAVS localized to the mitochondrial membrane.

HCV NS3/4A protease disrupts eYFP-MAVS/IFN-β-SEAP signaling pathway by proteolytic cleavage of eYFP-MAVS in a dose-dependent manner

Our assay was used to assess HCV replication in Huh7.5 cells that stably expressed full-length HCV replicons. The replicon cell lines were co-transfected with eYFP-MAVS and IFN-β-SEAP, and naive Huh7.5 cells were simultaneously transfected to serve as a control. SEAP activity in HCV replicon cells was approximately 20% relative to that in the control group ($P < 0.05$, Figure 2A). In the presence of HCV NS3/4A protease, eYFP-MAVS was proteolytically cleavaged as reported previously[11,16]. The proteolytically cleaved eYFP-MAVS, named ΔeYFP-MAVS, only could be detected in HCV replicon cells (Figure 2C), whose localization shifted from the mitochondrial membrane to the cytoplasm (Figure 2B).

The sensitivity of this assay was examined by co-transfecting eYFP-MAVS and IFN-β-SEAP with various concentrations of pNS3/4A, or with the control empty vector. SEAP activity was evaluated 24, 48 and 72 h post-transfection. The expression of NS3/4A protease in trans-

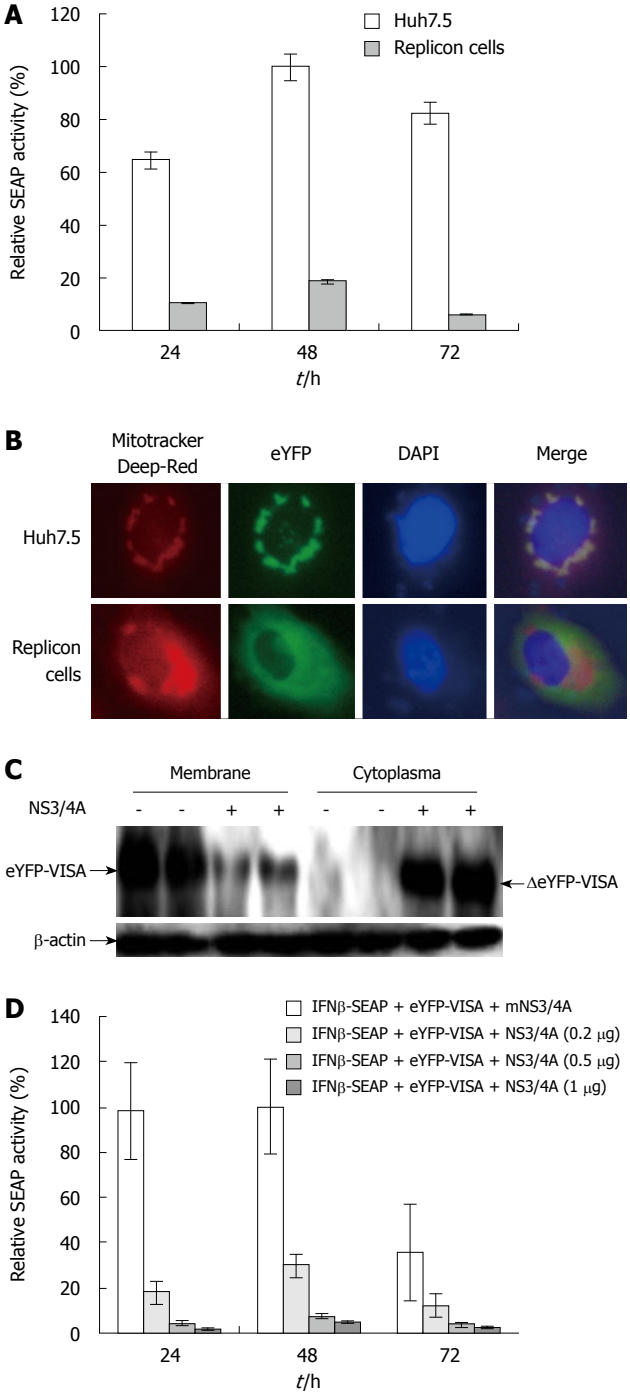

Figure 2 Hepatitis C virus NS3/4A protease activity impairs the enhanced yellow fluorescent protein-mitochondrial antiviral signaling protein/interferon-β-secreted placental alkaline phosphatase signaling pathway. A: Validation of the reporter assay system in Huh7.5 cells that contain full-length hepatitis C virus (HCV) replicons ($P < 0.05$). Huh7.5 and replicon cells were co-transfected with enhanced yellow fluorescent protein (eYFP)-mitochondrial antiviral signaling protein (MAVS) and interferon (IFN)-β-secreted placental alkaline phosphatase (SEAP). pRL-TK was co-transfected to normalize transfection efficiency. SEAP activity was examined at 24, 48 and 72 h after transfection. Bars indicate SD ($n = 3$); B: Localization of eYFP-MAVS. Subcellular localization of eYFP-MAVS was assessed by fluorescence microscopy 48 h post-transfection in Huh7.5 and replicon cells; C: Western blotting analysis of eYFP-MAVS cleaved by NS3/4A protease. Lysates of Huh7.5 and replicon cells treated as above were harvested at 48 h post-transfection and analyzed by Western blotting. Arrows indicate the positions of eYFP-MAVS and Δ eYFP-MAVS, respectively; D: Huh7.5 cells were co-transfected with eYFP-MAVS, IFN-β-SEAP and increasing amounts of expression plasmid pNS3/4A that encoded HCV NS3/4A protease (0, 0.2, 0.5 and 1 μg). pRL-TK was co-transfected to normalize transfection efficiency. SEAP activity in cell culture was measured at 24, 48 and 72 h post-transfection. Bars indicate SD ($n = 3$).

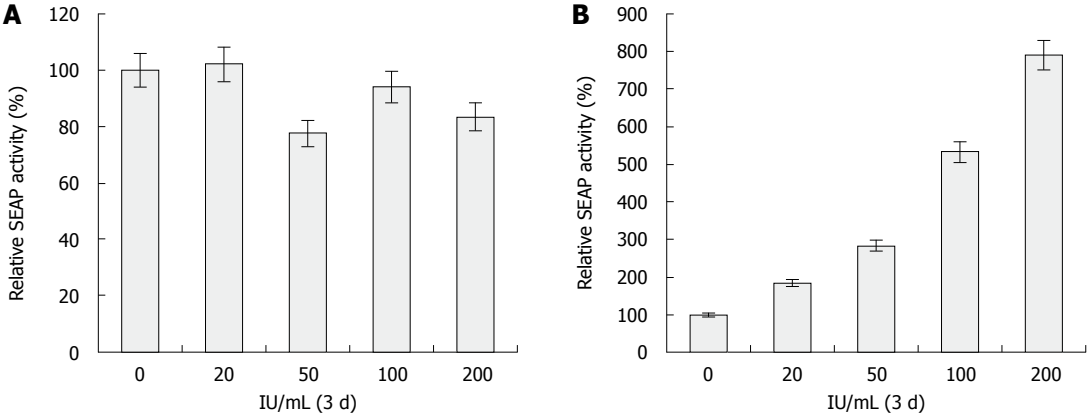

Figure 3 Secreted placental alkaline phosphatase reporter correlates with the anti-hepatitis C virus activity of interferon-α. A, B: Increase of secreted placental alkaline phosphatase (SEAP) activity in enhanced yellow fluorescent protein (eYFP)-mitochondrial antiviral signaling protein (MAVS) and interferon (IFN)-β-SEAP co-transfected Huh7.5 ($P > 0.05$) and replicon cells ($P < 0.05$) treated with IFN-α. Before transfection, Huh7.5 and replicon cells were incubated in the absence or presence of 20, 50, 100 and 200 IU/mL IFN-α for 72 h. SEAP activity was measured at 48 h post-transfection in the presence of IFN-α. The percentage increase in luciferase activity relative to the untreated controls was plotted. Bars indicate SD ($n = 3$).

fected cells resulted in the expected downregulation of the eYFP-MAVS/IFN-β-SEAP signaling pathway in a dose-dependent manner ($P < 0.05$, Figure 2D). These results indicated that this reporter system could be used for quantitative analysis of NS3/4A protease activity.

Feasibility of this system to reflect the activity of HCV inhibitors

To show the feasibility of this reporter system for characterizing HCV inhibitors, Huh-7.5 and HCV replicon cells incubated with various concentrations of IFN-α, ranging from 0 to 200 U/mL for 72 h were transfected with eYFP-MAVS and IFN-β-SEAP. At 48 h, SEAP activity of replicon cells was proportional to the extent of anti-HCV treatment ($P < 0.05$, Figure 3A). Meanwhile, Western blotting analysis of these samples revealed the amount of NS3 protease that was encoded by the HCV replicon was proportionally reduced by treatment with increasing concentrations of IFN-α (Figure 3B). The increase in SEAP activity was inversely proportional to the dose of active NS3/4A protease. No difference was observed with Huh-7.5 cells. These results indicate that this reporter system could be used for quantitative analysis of HCV-inhibitory compounds in cell culture systems.

Inhibitory effect of CsA on HCV replication and its combination effect with IFN-α

CsA is a well-characterized anti-HCV reagent that strongly suppresses viral replication in cell culture[17-19]. We evaluated the inhibitory effect of CsA on HCV replication and its combination effect with IFN-α. Huh-7.5 and replicon cells treated with increasing doses of CsA (Figure 4A) for 48 h were transfected with eYFP-MAVS and IFN-β-SEAP. At 24 h post-transfection, cells treated with 0.4 and 0.6 μg/mL CsA had significantly higher SEAP activity compared with cells treated with vehicle or 0.05 and 0.2 μg/mL CsA, whereas no difference was observed with Huh-7.5 cells (Figure 4A). As shown in Figure 4B, IFN-α in combination with CsA therapy enhanced its inhibitory effect. Cells treated with both agents had significant enhancement in SEAP activity compared with cells treated with a single agent. Determination of SEAP activity revealed that 0.2 μg/mL CsA-treated cells had a minimal increase in SEAP activity compared to untreated cells (Figure 4A), whereas, in combination with IFN-α, 0.2 μg/mL CsA-treated cells had a 5-6-fold increase. Cells treated with CsA and IFN-α showed a > 7-fold increase in SEAP activity.

DISCUSSION

To date, the only available therapy for chronic hepatitis C is IFN-α, either alone or in combination with the nucleoside analogue ribavirin. However, many patients are discouraged from IFN-based treatment because of severe side effects. In the past few years, much progress has been made in the development of small-molecule-based therapeutics for the treatment of HCV infection, such as inhibitors of HCV enzymes, as well as nucleic-acid-based agents that attack the viral RNA. However, the lack of appropriate model systems for drug evaluation has hampered their clinical use.

Although the replicon systems and the infectious HCV cell culture system have served as extremely valuable tools for *in vitro* study of HCV replication, and for screening and evaluation of new antiviral drugs against HCV that specifically target the protease activity of NS3 or the polymerase activity of NS5[20-26], the level of viral multiplication is not satisfactory, because in all cases, the detection of viral RNA and protein has to rely on reverse transcriptase-polymerase chain reaction and antibody labeling, which have some inherent technical problems that are difficult to overcome. To date, many different HCV replicons have been generated with other reporter genes such as firefly luciferase or fluorescent proteins, which allow screening of a high number of compounds in a fast and reproducible way[27-31]. However, the results from these systems should be interpreted with care because some adaptive mutations

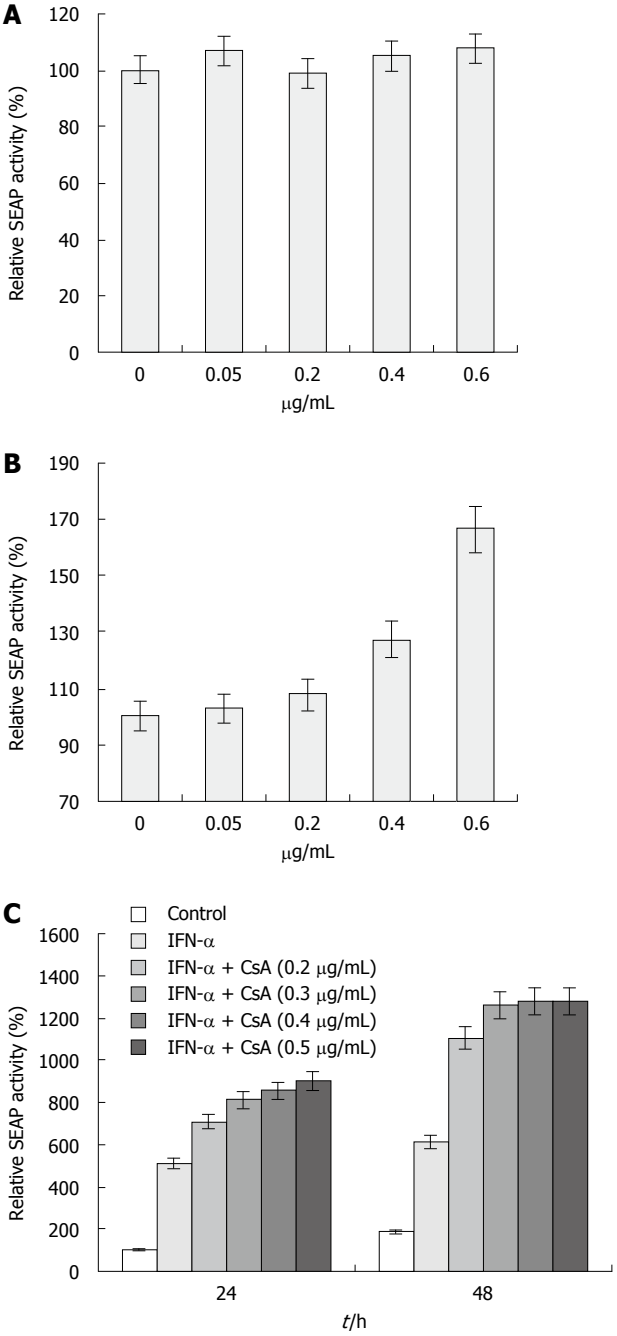

Figure 4 Inhibitory effect of cyclosporine A on hepatitis C virus replication and its combination effect with interferon-α. A, B: Increase of secreted placental alkaline phosphatase (SEAP) activity in enhanced yellow fluorescent protein (eYFP)-mitochondrial antiviral signaling protein (MAVS) and interferon (IFN)-β-SEAP co-transfected Huh7.5 ($P > 0.05$) and replicon ($P < 0.05$) cells treated with cyclosporine A (CsA). Before transfection, Huh7.5 and replicon cells were incubated with CsA at 0, 0.05, 0.2, 0.4 and 0.6 μg/mL for 48 h. SEAP activity was measured at 24 h post-transfection in the presence of CsA. The percentage increase in SEAP activity relative to the untreated controls was plotted. Bars indicate SD ($n = 3$); C: IFN-α in combination with CsA therapy enhanced its inhibitory effect. Replicon cells were incubated with IFN-α (100 IU/mL) in combination with CsA at 0, 0.2, 0.3, 0.4 and 0.5 μg/mL for 48 h, and co-transfected with eYFP-MAVS and IFN-β-SEAP; pRL-TK was co-transfected to normalize transfection efficiency. SEAP activity was measured at 24 and 48 h post-transfection in the presence of IFN-α and CsA. The percentage increase of SEAP activity relative to the untreated controls was plotted. Bars indicate SD ($n = 3$).

that mediate efficient replication *in vitro* might result in non-replicative replicons *in vivo*.

It has been previously reported that HCV NS3/4A protease cleaves MAVS from mitochondria to block the activation of IFN-β and nuclear factor-κB and the induction of antiviral effector genes in infected cells. In the present study, using the eYFP-MAVS/IFN-β-SEAP signaling pathway and HCV replicon model, we established a rapid and sensitive cell-based assay system to identify regulators of HCV replication. It allowed us to monitor HCV replication both qualitatively and quantitatively, using an easy, rapid and inexpensive system. HCV replication can be measured by the reporter enzyme SEAP, which enabled us to perform ongoing monitoring of anti-HCV drugs through repeated chemiluminescence. The cell lysates need not be prepared in our assay, and the SEAP protein is very stable and highly sensitive in enzymatic assays. SEAP activity in culture medium obtained from control cells transfected with eYFP-MAVS and IFN-β-SEAP was approximate 700 times greater than that obtained from replicon cells. Another reporter, eYFP-MAVS, which was localized to the mitochondrial membrane, but in the presence of HCV NS3/4A protease, it was proteolytically cleaved and shifted from the mitochondrial membrane to the cytoplasm, which can be dynamically monitored by fluorescence microscopy. Time- and dose-dependent studies have revealed that a decrease of SEAP activity and cleavage of eYFP-MAVS occur under conditions in which NS3/4A is active. Valuation of this reporter assay was verified through detecting the activity of IFN-α and CsA, which have been reported to be capable of modulating HCV replication. Although not investigated here, we believe that this reporter system could be of significant benefit in studies of HCV infection. This system will enable development or enhancement of therapeutic protocols by providing a platform to investigate dosing, scheduling, or the efficacy of combination therapies.

COMMENTS

Background

Advances in hepatitis C virus (HCV) replicon and infectious cell models provide systems for screening compounds against HCV. However, methods for detection of HCV protein or RNA require fixation or cell lysis, such as immunofluorescence, Western blotting and quantitative reverse transcriptase-polymerase chain reaction, and are insensitive or complex and time-consuming.

Research frontiers

Overexpression of mitochondrial antiviral signaling protein (MAVS) strongly activates interferon (IFN)-β promoter. MAVS self-association mediates innate immune signaling that can be blocked by HCV NS3/4A cleavage. Recent studies have highlighted the mechanism by HCV escapes the innate antiviral immunity mediated by HCV NS3/4A cleavage of MAVS. In this study, the authors developed a sensitive, simple assay for screening compounds against HCV based on HCV NS3/4A cleavage of the MAVS/IFN-β signaling pathway.

Innovations and breakthroughs

Although many studies have focused on the mechanism by which HCV escapes the innate antiviral immunity mediated by HCV NS3/4A blockage of the MAVS/IFN-β signaling pathway, its application for HCV detection has seldom been studied. The assay reported here based on HCV NS3/4A blockage of the MAVS/IFN-β signaling pathway converts the theoretical research into applied science. The reporter system provides a quantitative approach for detection of HCV replication based on measurement of secreted reporters in the medium, which is more sensitive and simpler compared with the methods described in background.

Applications

The reporter system here is a simple, sensitive and quantitative assay for anti-

HCV compounds and will provide another alternative efficient means for screening HCV inhibitors.

Terminology

Measurement of reporter expression level regulated by IFN-β promoter is an indicator for IFN-β promoter activation. Overexpression of MAVS strongly activates IFN-β-promoter-regulated reporter expression. However, in the presence of HCV NS3/4A, IFN-β promoter activation induced by MAVS was inhibited. By contrast, compounds against HCV will recover IFN-β promoter activation induced by MAVS.

Peer review

Fu *et al* have described concisely an HCV replicon model that is suitable to test the efficacy of anti-HCV compounds by means of checking the activity of the MAVS/IFN-β pathway. The materials and methods are clearly presented, although they could be explained in more detail. Results are demonstrative and conclusive and the English is also good, with only minor changes being necessary.

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BRIEF ARTICLE

Significant association between ABO blood group and pancreatic cancer

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Abstract

AIM: To evaluate whether the ABO blood group is related to pancreatic cancer risk in the general population of the United States.

METHODS: Using the University of Pittsburgh's clinical pancreatic cancer registry, the blood donor database from our local blood bank (Central Blood Bank), and the blood product recipient database from the regional transfusion service (Centralized Transfusion Service) in Pittsburgh, Pennsylvania, we identified 274 pancreatic cancer patients with previously determined serological ABO blood group information. The ABO blood group frequency was compared between these patients and 708 842 individual, community-based blood donors who had made donations to Pittsburgh's Central Blood Bank between 1979 and 2009.

RESULTS: The frequency of blood group A was statistically significantly higher amongst pancreatic cancer patients compared to its frequency amongst the regional blood donors [47.63% *vs* 39.10%, odds ratio (OR) = 1.43, P = 0.004]. Conversely, the frequency of blood group O was significantly lower amongst pancreatic cancer patients relative to the community blood donors (32.12% *vs* 43.99%, OR = 0.60, P = 0.00007). There were limited blood group B (n = 38) and AB (n = 17) pancreatic cancer patients; the overall P trend value comparing patient to donor blood groups was 0.001.

CONCLUSION: The ABO blood group is associated with pancreatic cancer risk. Future studies should examine the mechanism linking pancreatic cancer risk to ABO blood group.

Key words: ABO blood group; Pancreatic adenocarcinoma; Surveillance; Risk reduction; Epidemiology

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INTRODUCTION

Pancreatic adenocarcinoma is the fourth most common cancer in the United States among men and women and has the highest case-fatality rate of any of the major cancers, due to its late stage at diagnosis and poor response to traditional therapies[1]. The median survival of pancreatic cancer patients is about 6 mo, and the majority of patients do not have resectable disease at the time of their diagnosis[2]. Pancreatic cancer increases in likelihood with advancing age and predominates in certain ethnic/racial groups, such as African-Americans, Ashkenazi Jews, Pacific Islanders, and the New Zealand Maori[3]. The elevated risk among certain populations appears to be multifactorial in nature and likely is due to a combination of environmental and inherited factors. The most significant genetic variables that are associated with the development of pancreatic cancer remain to be identified, although various cancer syndromes and hereditary pancreatitis place individuals at significantly increased risk[4]. Cigarette smoking, chronic pancreatitis, diabetes mellitus and obesity are well-established risk factors for pancreatic cancer[5].

Mounting evidence has demonstrated that the ABO blood group may also be associated with pancreatic cancer. A Spanish study of 108 cases and 374 controls found a non-significantly elevated risk of pancreatic cancer among individuals with blood group A [relative risk (RR) = 1.52, 95% confidence interval (CI): 0.87-2.67][6]. An Italian series of 224 patients with histologically-confirmed pancreatic adenocarcinoma compared the ABO blood group distribution with two control groups: 7086 patients with various diseases (Group 1) and 7320 voluntary blood donors (Group 2). The researchers noted an increased incidence of pancreatic cancer among the blood group B patients (RR = 1.5 *vs* Group 1, $P = 0.021$; RR = 1.7 *vs* Group 2, $P = 0.0025$) and a decreased number in blood group O patients, when compared with the two control groups[7]. Additionally, a combined, prospective cohort study of over 107 000 US health professionals observed that blood groups A, B, and AB were associated with an overall increased risk of developing pancreatic cancer[8]. More recently, a genome-wide association study (GWAS) of 1896 individuals with pancreatic cancer and 1939 controls - validated using an additional 2457 affected individuals and 2654 controls - identified a single nucleotide polymorphism (SNP) in intron 1 of the *ABO* gene (rs505922) as a genetic risk factor for pancreatic cancer [odds ratio (OR) = 1.20; 95% CI: 1.12-1.28][9]. In an effort to further discern how pancreatic cancer and the ABO blood group might be associated, we investigated the relationship of pancreatic cancer incidence with ABO blood group in our Western Pennsylvania regional population. We compared the serologically-determined ABO blood group of pancreatic cancer patients with the ABO group of greater than 700 000 blood donors to the Central Blood Bank in Pittsburgh.

MATERIALS AND METHODS

To estimate the ABO frequencies in our local area, the donor database of the Central Blood Bank in Pittsburgh, Pennsylvania was queried for the ABO group of individual donors. The Central Blood Bank collects blood mainly in southwestern Pennsylvania. This database includes all individuals who have made a blood donation to the Central Blood Bank over the past approximately 30 years. Between 1979 and 2009, there were 708 842 unique blood donors. Pancreatic cancer patients from the University of Pittsburgh's affiliated hospitals - Presbyterian University Hospital and the University of Pittsburgh Cancer Institute's Hillman Cancer Center - who had provided informed consent to be part of their pancreatic cancer research registry were included in this study. Of 359 eligible pancreatic cancer patients in the registry, 274 had a historical or current ABO blood group available after querying the Centralized Transfusion Service's database. The Centralized Transfusion Service's database includes all patients who have a type and screen performed at Pittsburgh area hospitals. Blood donors were screened for infectious diseases according to Food and Drug Administration (FDA) and American Association of Blood Banks (AABB) regulations. FDA approved manual and automated ABO grouping methods and reagents were employed both at the Central Blood Bank and at the Centralized Transfusion Service. Proportions of ABO blood groups for pancreatic cancer cases and regional blood donors were compared using Chi-squared analysis. Data analysis was performed using R Project software (www.r-project.org).

RESULTS

Table 1 shows the OR of the ABO blood group distribution of the 274 pancreatic cancer patients in comparison with the ABO groups of the 708 842 unique blood donors to the Central Blood Bank in the Pittsburgh area over approximately the past 30 years. The frequency of blood group A was statistically significantly higher amongst the pancreatic cancer patients compared to the frequency amongst the regional blood donors. Conversely, the frequency of blood group O was statistically significantly lower amongst the pancreatic cancer patients relative to the community blood donors.

Statistically significant associations were not revealed among the limited number of blood group B ($n = 38$) and AB ($n = 17$) pancreatic cancer patients and regional blood donors; the overall P trend value comparing patient to donor ABO blood groups was 0.001.

DISCUSSION

Our study demonstrates that pancreatic cancer patients

Table 1 Odds ratio of pancreatic cancer patients stratified by ABO group in relation to the ABO groups of regional unique blood donors

Blood group	Unique blood donors, *n* (%)	Pancreatic cancer patients, *n* (%)	Odds ratio *vs* other blood groups
Group A	277 133 (39.10)	131 (47.63)	1.43 (0.004)
Group B	87 252 (12.31)	38 (13.87)	1.15 (0.41)
Group AB	32 662 (4.61)	17 (6.20)	1.37 (0.19)
Group O	311 795 (43.99)	88 (32.12)	0.60 (0.000070)
Total	708 842	274	*P* trend patients *vs* donors = 0.001

treated at a large, US institution are significantly more likely to be blood group A than regional blood donors and are significantly less likely to be blood group O than regional blood donors; the proportions of patient *vs* donor ABO blood groups were statistically different from what would be expected by chance alone. We examined this relationship among over 700 000 unique individuals who made blood donations to the Central Blood Bank in Pittsburgh between 1979 and 2009. Although there was a trend for risk of pancreatic adenocarcinoma in association with blood groups B and AB, a statistically significant effect was not observed, likely due to limited study power for detecting differences in these small subpopulations of individuals.

In a prospective cohort study published in 2009 of 107 503 US residents derived from the Nurses' Health Study (77 360 eligible female nurses) and the Health Professionals Follow-up Study (30 143 eligible male health professionals), individuals with blood group A, B and AB were noted overall to have an elevated risk of pancreatic cancer compared to those participants with blood group O[8]. However, taking each study separately, only blood group B was associated with increased cancer risk. Notably, blood group was self-reported in that prospective study, although a validation analysis displayed a greater than 90% concordance rate between reported and actual ABO blood group. In our study, the presence of serologically-determined blood group information from the blood bank was an entry criterion; thus, there was no possibility of recall bias influencing our results.

The *ABO* locus is located on chromosome 9 and there are three main alleles in the system: *A101*, *B101*, and *O01*[10]. The *A101* allele encodes a glycosyltransferase that adds a terminal α-N-acetylgalactosamine to H antigen, producing the A antigen. Similarly the *B101* allele encodes a glycosyltransferase that adds a terminal α-D-galactose to H antigen, thus creating the B antigen. The most common types of *O* alleles contain a critical 1-bp deletion compared to the consensus *A101* allele and, if translated, would give rise to non-functional enzymes[10,11]. In a group O individual, the H antigen is not modified. In addition to studies of pancreatic cancer, numerous past studies have shown that blood group A is related to gastric cancer[12,13]. The precise biological reasons as to why there is a relationship between ABO blood group and certain cancers are unknown, although two recent GWAS demonstrated that particular SNPs at the *ABO* locus were associated with the inflammatory cytokines tumor necrosis factor[14] and intercellular adhesion molecule 1[15]. The ABO blood group has also been shown to be related to other biological processes; for example, although a definitive mechanism has not yet been elucidated, levels of von Willebrand factor (vWF) antigen have been statistically shown to correlate to the ABO group with group AB individuals demonstrating the highest average vWF levels and group O individuals having the lowest average vWF levels[16].

The ABO blood group is genetically-determined and therefore is not a modifiable risk factor as are cigarette smoking, body mass index, diet or other lifestyle-related variables. The risk of pancreatic cancer for individuals with blood group A has been replicated in several studies. Nonetheless, the magnitude of risk is not high enough to warrant clinical screening, especially considering that these approaches are still in the developing stages[17]. However, the risk of pancreatic cancer in individuals with blood group A (and likely B or AB) is nearly as strong as the risk of developing pancreatic cancer as a consequence of cigarette smoking; thus, the combination of multiple moderate risk factors (such as age and family history[18]) could be used to calculate whether some individuals are at high enough risk to warrant counseling for risk reduction strategies or inclusion in pancreatic cancer screening trials.

The importance of the ABO blood group in assessing pancreatic cancer risk is also highlighted by the lack of other global genetic risk factors identified in the GWAS by Amundadottir *et al*[9]. This study indicates that the etiology of pancreatic cancer is more complex than previously believed and likely represents an array of deleterious pathways. Although the mechanisms of pancreatic cancer oncogenesis have not been fully deciphered, the association of ABO blood group with pancreatic cancer risk has been confirmed.

COMMENTS

Background

Pancreatic adenocarcinoma has the highest case-fatality rate of any of the major cancers. Because there are no accepted population-based screening methods, identifying individuals at greatest risk of developing this lethal form of cancer is paramount to developing risk reduction strategies. Previous evidence has indicated that ABO blood group may influence the risk of developing pancreatic cancer.

Research frontiers

Blood groups A, B, and/or AB have been associated with pancreatic cancer in a limited number of past studies. Genome-wide association studies have also identified a risk locus in the *ABO* gene.

Innovations and breakthroughs

This study compared the frequency of ABO blood groups in a cohort of pancreatic cancer patients to the blood group distributions in greater than 700 000 community-based blood donors. The authors demonstrated that blood group A was statistically significantly higher and blood group O was significantly less frequent among pancreatic cancer patients than in community blood donors. In contrast to many past studies, the authors used serologically-confirmed blood group information.

Applications

ABO blood group information is valuable in predicting who may be at risk of developing pancreatic adenocarcinoma and should be incorporated into risk stratification and surveillance studies.

Peer review

The authors report an interesting study about the connection of ABO blood group and the development of pancreatic cancer. The paper is written well and good to read, the data presented clearly and the whole paper of relevance.

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BRIEF ARTICLE

Linear endoscopic ultrasonography *vs* magnetic resonance imaging in ampullary tumors

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Abstract

AIM: To assess linear endoscopic ultrasound (L-EUS) and magnetic resonance imaging (MRI) in biliary tract dilation and suspect small ampullary tumor.

METHODS: L-EUS and MRI data were compared in 24 patients with small ampullary tumors; all with subsequent histological confirmation. Data were collected prospectively and the accuracy of detection, histological characterization and N staging were assessed retrospectively using the results of surgical or endoscopic treatment as a benchmark.

RESULTS: A suspicion of ampullary tumor was present in 75% of MRI and all L-EUS examinations, with 80% agreement between EUS and histological findings at endoscopy. However, L-EUS and histological TN staging at surgery showed moderate agreement (κ = 0.54).

CONCLUSION: L-EUS could be a useful adjunct as a diagnostic tool in the evaluation of patients with suspected ampullary tumors.

Key words: Ampullary tumors; Endoscopic ultrasound; Magnetic resonance imaging

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INTRODUCTION

Ampullary tumors are infrequent entities that represent about 0.2% of gastrointestinal malignancies[1]. However, these neoplasms are considered as diagnostic challenges, because they display a wide array of pathological features, from mild dysplasia to high-grade dysplasia and invasive

carcinoma[2]. Clinical presentation includes vague abdominal pain, liver enzyme elevation, jaundice, recurrent pancreatitis, or uncommon symptoms such as gastrointestinal bleeding or duodenal obstruction[3,4].

Although endoscopic papillectomy represents a possible treatment[5], most ampullary tumors still undergo a surgical approach[6,7]. Thus, the diagnostic evaluation must be as careful as possible[8], because ampullary carcinoma is difficult to diagnose at an early stage and multiple imaging techniques should be carried out appropriately to establish a diagnosis and improve prognosis[9]. In fact, neither definitive methods for early diagnosis nor specific markers are available for this disease[10].

In recent years, endoscopic ultrasound (EUS) has been shown to be superior to computed tomography (CT)[11] and conventional ultrasound scans[12], and equivalent to magnetic resonance imaging (MRI) for tumor detection and T and N staging of ampullary tumors[13].

Echoendoscopes are classified into radial and linear instruments[14]: to date, almost all information available on detection and staging of ampullary tumors has been obtained with radial echoendoscopes, and there are no studies with linear EUS (L-EUS) for this purpose.

The aim of the present study was to compare L-EUS and MRI in the diagnostic evaluation of patients with suspected ampullary neoplasms.

MATERIALS AND METHODS

In a consecutive series of 1205 L-EUS biliopancreatic examinations carried out in the period July 2007 to August 2009, there were 44 symptomatic patients (referred for increasing liver enzymes, jaundice, abdominal pain or dilation of the biliary tract) who were evaluated for suspicion of ampullary tumors. In 20 of these, the ampullary tumor was excluded by L-EUS that revealed other causes for their symptoms (four stones, four mediocholedocal stenoses, and 12 pancreatic cephalic small cancer). In the remaining 24 patients, data were collected prospectively and the accuracy of detection, histological characterization, and N staging were assessed retrospectively using the surgical or endoscopic results as a benchmark.

The following inclusion criteria were adopted: (1) cholestatic syndrome with previous negative or uncertain conventional US and CT imaging; (2) absence of previously known biliary and/or pancreatic diseases; (3) absence of advanced ampullary tumors; (4) histological diagnosis of the resected specimen; (5) MRI (axial, coronal and radial sequences, T2 single-shot, performed by a radiologist with experience of the biliary tree); and (6) L-EUS. Exclusion criteria included previously known biliary or pancreatic disease, and the presence of evident, large ampullary tumors at endoscopy.

L-EUS was carried out by means of a linear array echoendoscope (Pentax EG 33830UT or Pentax EG 3870UTK; Hamburg, Germany) that was inserted into the second part of the duodenum after intravenous midazolam and meperidine titrated to obtain conscious sedation. Keeping the tip of the echoendoscope in touch with the duodenal mucosa, the echoendoscope was torqued counterclockwise and slowly withdrawn into the duodenal bulb[14], and after visualization of the papilla of Vater, its endoscopic aspect was considered and recorded.

Ampullary carcinoma visualized by EUS was staged according to the TN classification[15]: T1 if the tumor echo was limited to the main duodenal papilla; T2 if the tumor echo invaded the duodenal muscularis propria layer; T3 if the tumor echo invaded the pancreas; and T4 if the tumor echo invaded peripancreatic soft tissues or other adjacent organs or vascular structures. EUS criteria for lymph node metastasis, classified as N1, were circularity, at least 10 mm in size, and hypoechogenicity.

Ethical considerations

This was a retrospective study and no study-driven clinical intervention was performed. Simplified Institutional Review Board approval for retrospective studies was obtained.

Statistical analysis

Differences in percentage of detection of ampullary tumors between MRI and L-EUS and between L-EUS and histology were assessed by the χ^2 test. Values of $P < 0.05$ were chosen for rejection of the null hypothesis. Moreover, a κ value for agreement between endoscopic and surgical histology and L-EUS and histological TN staging was calculated. The value was scored according to standard criteria[16].

RESULTS

Data from 24 patients (17 men and seven women, aged 60 ± 12 years, range: 42-88 years) fulfilled the entry criteria and were evaluated. Demographic data, referral reasons, clinical, radiological and histological features are summarized in Tables 1 and 2. Abdominal pain was the common symptom in all patients; jaundice was present in 13 (54%) and liver enzyme elevation was detected in 12 (50%). One patient was evaluated due to dilation of both extrahepatic and main pancreatic ducts. Multiple endoscopic forceps biopsies were taken from all cases during L-EUS evaluation. In one case, it was impossible to analyze the sample due to material not being available in the test-tube. Agreement between endoscopic and histological results was very good ($\kappa = 0.81$).

All patients underwent surgery except for two (stage T1N0) who were treated by endoscopic ampullectomy, and histological results were available for the entire group (Figure 1). Average diameter of the ampulla was 2 ± 0.8 cm (range: 1.5-2.6 cm).

MRI examination was negative in 6 (25%) cases, showed indirect signs (dilatation of intrahepatic and extrahepatic bile ducts) of space occupying lesions of the ampulla in

Table 1 Demographic data and reasons for referral of patients with ampullary tumors

Patient No.	Sex/age (yr)	Referral reason
1	M/53	Liver enzymes elevation + jaundice
2	F/47	Abdominal pain
3	M/71	Liver enzymes elevation + jaundice
4	M/62	Liver enzymes elevation + jaundice
5	F/53	Liver enzymes elevation
6	F/67	Liver enzymes elevation
7	M/45	Liver enzymes elevation
8	M/73	Liver enzymes elevation
9	M/80	Liver enzymes elevation
10	F/44	Liver enzymes elevation
11	F/50	Liver enzymes elevation + jaundice
12	M/48	Liver enzymes elevation + jaundice
13	M/66	Liver enzymes elevation
14	M/67	Liver enzymes elevation + jaundice
15	M/59	Liver enzymes elevation
16	M/67	Liver enzymes elevation + jaundice
17	F/60	Liver enzymes elevation
18	M/63	Liver enzymes elevation + jaundice
19	F/54	Liver enzymes elevation + jaundice
20	M/65	Liver enzymes elevation
21	M/64	Liver enzymes elevation + jaundice
22	M/42	Liver enzymes elevation + jaundice
23	M/47	Liver enzymes elevation + jaundice
24	F/88	Liver enzymes elevation + jaundice

17 (71%) cases, and an actual space occupying lesion of the ampulla in 1 (8%) case (Figure 2).

L-EUS detected ampullary tumors in all 24 (100%) patients ($P < 0.03$ *vs* MRI) (Figure 2).

In 19 (80%) cases, Histological analysis revealed intestinal-type adenocarcinoma in 19 (80%) cases (stage T2N0 in 14 and T2N1 in five) CDX2 positive, adenoma with high-grade dysplasia in 2 (8%), and adenoma with low-grade dysplasia in the remaining 3 (12%) (Figure 3).

No biliary/pancreatic-type tumors were found. Thus, L-EUS was able to detect malignant lesions in 87% of ampullary lesions ($P = 0.21$ *vs* histological results), with sensitivity of 87.5% and specificity of 100% and a moderate agreement ($\kappa = 0.54$) between L-EUS and TN histological staging.

DISCUSSION

Although MRI is regarded as the most reliable noninvasive diagnostic imaging modality for the evaluation of pancreatobiliary lesions[17,18], and is considered as a substitute for diagnostic endoscopic retrograde cholangiopancreatography[19], it cannot provide biopsy samples and rarely identifies whether the obstruction is benign or malignant, especially when lesions are small. The ampulla of Vater is a possible blind spot for MRI because of its small size and the tapering of the intramural ducts that contain little fluid[9,18,20]. Thus, other investigative modalities have been added to the diagnostic armamentarium; among these, EUS has proved to be useful and reliable. Generally, radial EUS is considered the gold standard[17,18] whereas L-EUS performance has never been studied.

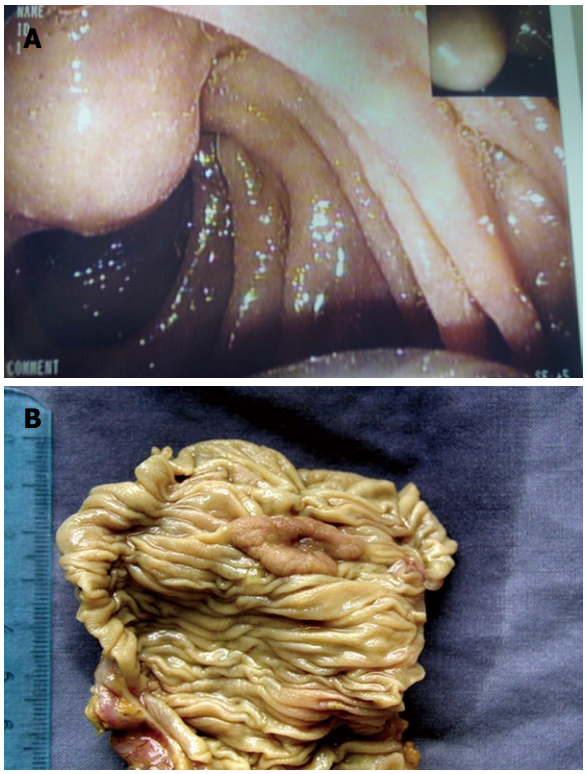

Figure 1 Endoscopic aspect (A) and resected surgical specimen (B) of a small ampullary tumor.

This is believed to be the first study to report L-EUS as a useful diagnostic tool for detection and staging of small ampullary tumors. In our experience, also supported by histological findings, this technique was able to raise a suspicion of ampullary neoplasm when other imaging techniques, including MRI, were not. Indeed, a suspicion of ampullary neoplasms was observed in 75% of MRI investigations compared with 100% of L-EUS scanning; the latter proved to be accurate, identifying > 80% of these lesions as malignant. The accuracy of EUS in the TNM staging of ampullary tumors remains controversial[17,21]. Histological grade is the gold standard, with the possibility to differentiate between ampullary tumor of intestinal type and those originating from the biliary or pancreatic ducts; however, it is worthy of note that a discrete correlation was found between L-EUS and histological TN staging in the present study. The discrepancies were due more to overstaging in 6 (25%) patients than to understaging in 3 (12%) patients. Overstaging can occur in the presence of peritumoral inflammation, whereas understaging can occur in the presence of minimal malignant infiltration of the pancreas[9,22]. Other recent experience also suggests that EUS is an accurate diagnostic test and exhibits a high level of agreement with surgical pathology[23].

However, L-EUS provided several advantages compared to MRI, such as the possibility of obtaining direct endoscopic visualization of the major duodenal papilla, and to depict the layered structures of the periampullary area. Thus, it is often possible to give a judgment on a pos-

Table 2 Clinical-radiological variables and histological features of ampullary tumors

Patients	US	CT-scan	MRI	L-EUS	Endoscopic imaging + biopsies	Surgery	Definitive staging and histology
1	Not done	Neg	Suspicion:dilation CBD + WD	T2N0	Visible lesion 1.6 cm-HGD	DCP	PT2N0, ADK
2	Uncertain	Neg	Suspicion:dilation CBD + WD	T2N0	Visible lesion 2.0 cm-HGD	DCP	PT2N1, ADK
3	Not done	Neg	Suspicion:dilation CBD + WD	T2N0	Visible lesion 1.8 cm-HGD	DCP	PT2N0, ADK
4	Not done	Not done	Suspicion:dilation CBD + WD	T2N0	Visible lesion 2.0 cm -HGD	DCP	PT2N0, ADK
5	Not done	Not done	Suspicion:dilation CBD + WD	T2N0	Visible lesion 1.5 cm-HGD	DCP	PT2N0, ADK
6	Neg	Neg	Neg	T1N0	Visible lesion 2.0 cm-LGD	EMR	PT1N0, LGD
7	Not done	Neg	Neg	T1N0	Visible lesion 2.0 cm-LGD	EMR	PT1N0, LGD
8	Neg	Not done	Neg	T2N0	Visible lesion 2.0 cm -HGD	SA	PT1N0, HGD
9	Neg	Neg	Suspicion:dilation CBD	T2N0	Visible lesion 2.0 cm-ADK	DCP	PT2N0, ADK
10	Neg	Neg	Neg	T2N0	Visible lesion 2.0 cm HGD	DCP	PT2N0, ADK
11	Neg	Neg	Suspicion:dilation CBD + WD	T2N0	Visible lesion 2.0 cm-ADK	DCP	PT2N0, ADK
12	Neg	Neg	Suspicion:dilation CBD + WD	T2N0	Visible lesion 2.6 cm-ADK	DCP	PT2N1, ADK
13	Not done	Neg	Neg	T1N0	Visible lesion 1.5 cm-LGD	SA	PT1N0, LGD
14	Neg	Not done	Suspicion:dilation CBD	T2N0	Visible lesion 2.5 cm-ADK	DCP	PT2N0, ADK
15	Neg	Not done	Suspicion:dilation CBD	T2N1	Visible lesion 2.0 cm-HGD	DCP	PT2N0, ADK
16	Neg	Uncertain	Suspicion:dilation CBD	T2N1	Visible lesion 2.0 cm-HGD	DCP	PT2N0, ADK
17	Neg	Neg	Suspicion:dilation CBD	T2N1	Visible lesion 2.0 cm-ADK	DCP	PT2N0, ADK
18	Neg	Neg	Suspicion:dilation CBD	T2N1	Visible lesion 1.5 cm-ADK	DCP	PT2N1, ADK
19	Not done	Neg	Suspicion:dilation CBD + WD	T2N1	Visible lesion 2.0 cm-ADK	DCP	PT2N1, ADK
20	Neg	Neg	Suspicion:dilation CBD	T1N0	Visible lesion 1.5 cm-HGD	DCP	PT2N0, ADK
21	Not done	Neg	Neg	T2N1	Visible lesion 2.0 cm-ADK	DCP	PT2N0, ADK
22	Not done	Not done	Suspicion:dilation CBD	T2N0	Visible lesion 2.5 cm-not available	DCP	PT1N0, HGD
23	Uncertain	Neg	Suspicion:dilation CBD + WD	T2N1	Visible lesion 2.5 cm-ADK	DCP	PT2N1, ADK
24	Neg	Neg	Suspicion:dilation CBD + WD	T2N1	Visible lesion 2.0 cm-ADK	SA due to age	PT2N0, ADK

ADK: Adenocarcinoma; CBD: Common bile duct; DCP: Duodeno-cephalo-pancreasectomy; HGD: High grade dysplasia; LGD: Low grade dysplasia; Neg: Negative for ampullary tumor; SA: Surgical ampullectomy; WD: Wirsung duct; US: Ultrasound; CT: Computed tomography; MRI: Magnetic resonance imaging; L-EUS: Linear endoscopic ultrasound; EMR: Endoscopic mucosal resection.

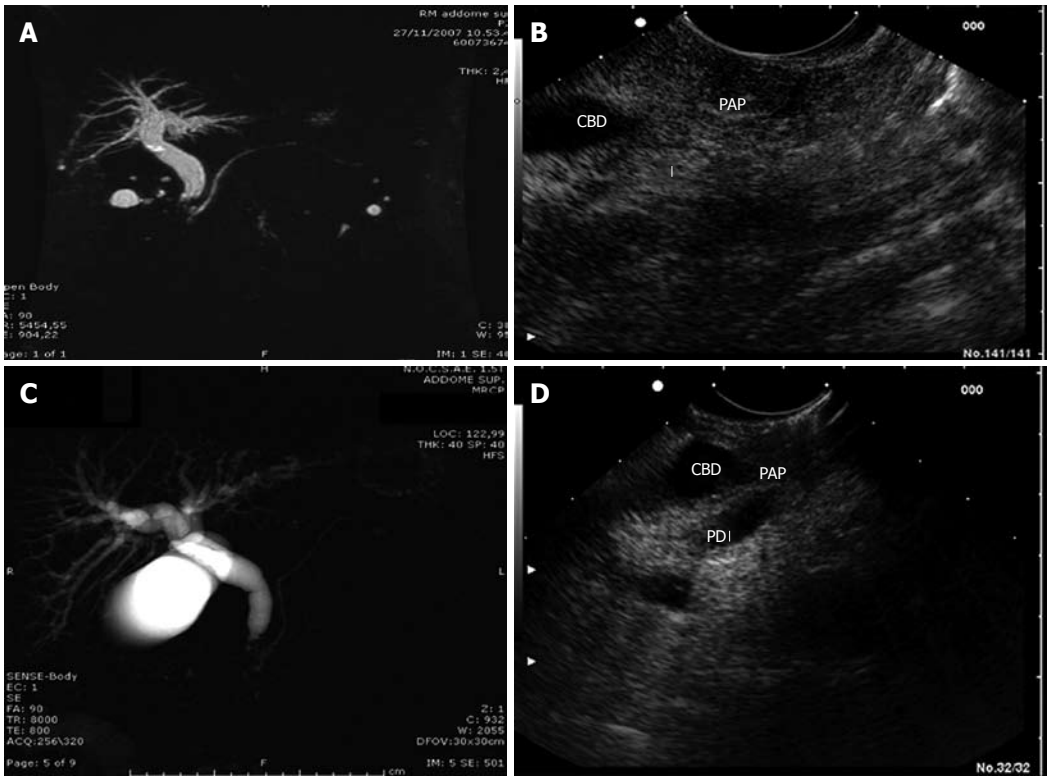

Figure 2 Ampullary tumor, stage 1. A: Magnetic resonance imaging (MRI) showed dilation of the common bile duct and Wirsung's duct, without space-occupying lesions of the ampulla; B: Linear endoscopic ultrasound (L-EUS) of the same patient. Ampullary tumor, stage 2; C: MRI showed dilation of the common bile duct and normal appearance of Wirsung's duct; D: L-EUS scan of the same patient, which showed duodenal wall disruption without pancreas invasion. CBD: Common bile duct; PAP: Papilla (Vater's papilla); PD: Pancreatic duct.

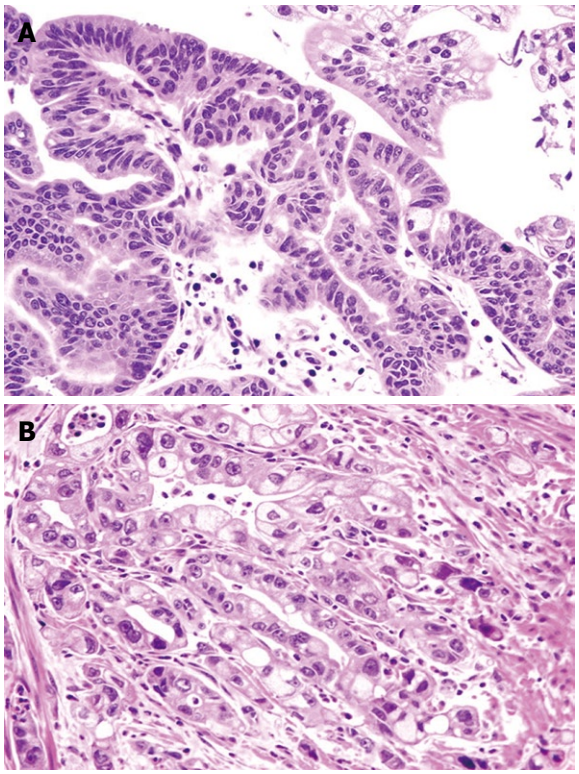

Figure 3 Ampullary tumor, low-grade dysplasia (A) and ampullary adenocarcinoma (B). Hematoxylin and eosin (HE), original magnification 40 ×.

sible endoscopic approach, because it also demonstrates intracholedochal growth. Limitations of this technique include inability to differentiate early malignant from benign tumors and to demonstrate distant metastases, in addition to being an invasive method.

The present study had some limitations. It was retrospective, the patient cohort was relatively small, and it was carried out in a setting with particular expertise in EUS. It remains to be established whether these results can be translated to a more general setting.

In conclusion, L-EUS appears to be a valid diagnostic tool to identify and stage small ampullary tumors, and might yield results that are superior to those of other diagnostic techniques. Further prospective studies are clearly needed to confirm these observations.

COMMENTS

Background

Ampullary tumors are rare and difficult to diagnose. The correct diagnosis is often reached after serological, radiological and/or endoscopic tests. Recently, endoscopic ultrasound (EUS) has been demonstrated as being as useful as magnetic resonance imaging (MRI) for detection of these neoplasms.

Innovations and breakthroughs

No previous study has been previously conducted with L-EUS in this setting, and this study by Manta *et al* showed that this appears to be a valid diagnostic tool to identify and stage small ampullary tumors, and might yield results that are superior to those with other diagnostic techniques.

Terminology

EUS and L-EUS: techniques that combine endoscopic and echographic approaches for detecting lesions of the gastrointestinal tract. MRI: a technique that employs nuclear resonance imaging to visualize abdominal sections without exposing the subject to radiation.

Peer review

This is a retrospective study with a small sample size. Despite this, it is a novel and well-designed study that is likely to influence clinical practice.

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BRIEF ARTICLE

Left-sided appendicitis: Review of 95 published cases and a case report

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Abstract

AIM: To give an overview of the literature on left-sided acute appendicitis (LSAA) associated with situs inversus totalis (SIT) and midgut malrotation (MM).

METHODS: We present a new case of LSAA with SIT and a literature review of studies published in the English language on LSAA, accessed *via* PubMed and Google Scholar databases.

RESULTS: Ninety-five published cases of LSAA were evaluated and a 25-year-old female, who presented to our clinic with left lower abdominal pain caused by LSAA, is reported. In the reviewed literature, fifty-seven patients were male and 38 were female with an age range of 8 to 82 years and a median age of 29.1 ± 15.9 years. Sixty-six patients had SIT, 23 had MM, three had cecal malrotation, and two had a previously unnoted congenital abnormality. Fifty-nine patients had presented to the hospital with left lower, 14 with right lower and seven with bilateral lower quadrant pain, and seven subjects complained of left upper quadrant pain. The diagnosis was established preoperatively in 49 patients, intraoperatively in 19, and during the postoperative period in five; 14 patients were aware of having this anomaly. The data of eight patients were not unavailable. Eleven patients underwent laparoscopic appendectomy, which was combined with cholecystectomy in two cases. Histopathological examination of the appendix specimens revealed adenocarcinoma in only two of 95 patients.

CONCLUSION: The diagnosis of left lower quadrant pain is based on well-established clinical symptoms, physical examination and physician's experience.

Key words: Diagnostic dilemma; Left lower quadrant pain; Left-sided appendicitis; Midgut malrotation; Situs inversus totalis

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INTRODUCTION

Acute appendicitis is probably the most common intraabdominal condition requiring emergency surgery. The diagnosis is based on well-established clinical symptoms, basic radiologic findings and surgeon experience[1,2]. Approxi-

mately one third of patients with acute appendicitis have pain localized outside of the right lower quadrant because of the various positions of the appendix vermiformis, i.e. retrocecal, pelvic, subcecal, preileal and postileal, while subhepatic, meso-celiac, mid-inguinal and left-sided are seen more rarely[1,2].

Appendicitis causing pain in the left lower quadrant is extremely rare and can occur with congenital abnormalities that include true left-sided appendix or as an atypical presentation of right-sided, but long appendix, which projects into the left lower quadrant[2]. Left-sided acute appendicitis (LSAA) develops in association with two types of congenital anomalies: situs inversus totalis (SIT) and midgut malrotation (MM)[1-65]. Herein, we report an unusual case of SIT with acute appendicitis presenting as left lower quadrant abdominal pain. We also reviewed 95 published cases of LSAA with congenital anomalies retrieved from the Google Scholar and PubMed databases.

MATERIALS AND METHODS

We report a new case of LSAA with SIT. Additionally, for the review, a search of the English medical language literature in PubMed and Google Scholar was conducted for every case report, series, letter to the editor, original article and literature review relating to left-sided appendicitis. In addition, reference lists of the articles obtained and previous reviews were examined. Key words used were left lower quadrant pain, LSAA, appendicitis with left lower quadrant pain, SIT and appendicitis, acute left-sided appendicitis, and MM and appendicitis. The search included all articles from 1893 to July 2010. The articles containing adequate information such as patient age, sex, localization of the symptoms, time of the diagnosis, type of congenital anomalies, choice of incision and surgery were included in the study, while studies and comment articles with insufficient clinical and demographic data were excluded.

RESULTS

Case report

A 25-year-old female presented to the emergency unit on June 18, 2010 with severe abdominal pain, which started the previous night. The patient stated that the pain has begun first in the epigastric area and later expanded through the left lower quadrant. Besides the pain, she complained of inappetence and mild nausea. Patient history revealed no previous illness or surgery. On physical examination, rebound tenderness was observed in the left lower quadrant. Laboratory tests, including leukocyte count (9100 K/UL), were normal. Based on the patient's clinical status and our previous experience, we considered LSAA in the differential diagnosis; accordingly, relevant analyses were performed. Chest X-ray revealed dextrocardia (Figure 1) and abdominal ultrasonography (USG) demonstrated SIT and blind intestinal loop consistent with acute appendicitis in the left lower quadrant. The patient was immediately taken to surgery and as the laparoscopic device was out of order, a left McBurney's incision was performed. She was discharged on the second postoperative day with no complications (Figure 2). Contrast-enhanced thoraco-abdominal computed tomography (CT) in the postoperative period confirmed the diagnosis of SIT (Figure 3).

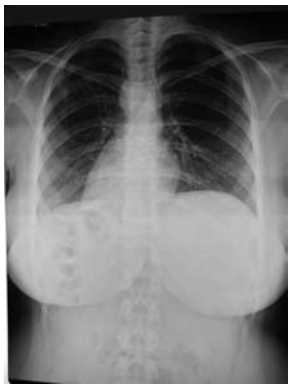

Figure 1 Chest X-ray showing dextrocardia.

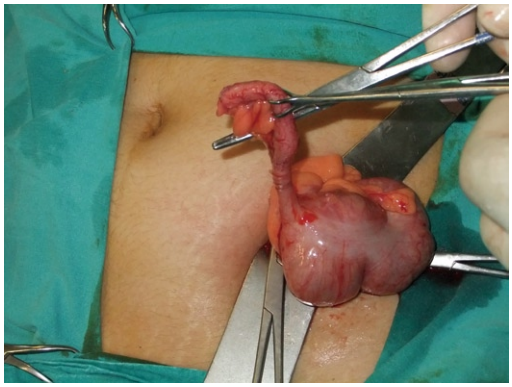

Figure 2 Intraoperative photograph showing an appendix together with cecum in the left lower quadrant.

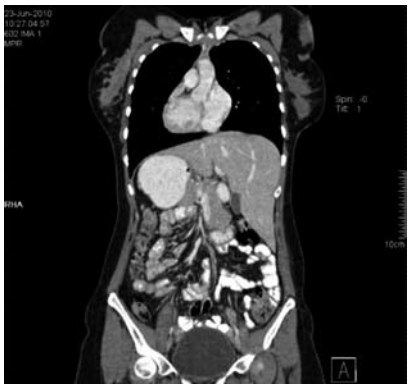

Figure 3 Coronal contrast-enhanced multidetector computed tomography of the thorax, abdomen and pelvis. Computed tomography showed situs inversus totalis including dextrocardia, right-sided gastric bubble and reversed spleen and liver (postoperative view).

Analysis of literature about left-sided appendicitis

The English medical literature published to July 2010 in PubMed and Google Scholar databases was reviewed, and 64 reports concerning 95 cases of LSAA meeting the above-

Table 1 Summary of 95 reported cases of left-sided acute appendicitis with situs inversus totalis and midgut malrotation *n* (%)

Patient characteristics	Results
Mean age (yr, range)	29.1 ± 15.9 (8-82)
Sex	
Male	57 (60)
Female	38 (40)
Pain location	
Left-lower quadrant	59 (62.1)
Right-lower quadrant	14 (14.7)
Bilateral lower quadrant	7 (7.3)
Pelvic	2 (2)
Left-upper quadrant	7 (7.3)
Peri-umbilical	6 (6.3)
Congenital anomaly	
Situs inversus totalis	66 (69.4)
Midgut malrotation	23 (24.2)
Cecal malrotation	3 (3)
Unnoted	2 (2)
Other	1 (1)
Time of diagnosis	
Preoperative	49 (51.5)
Intraoperative	19 (20)
Known	14 (14.7)
Postop	5 (5.2)
Unnoted	8 (8.4)

mentioned criteria were included in this review[1-64]. The article types were as follows: case report - 48, case report and a review of the literature - 7, imaging for surgeon - 5, letter to the editor - 2, and abstract - 2. The clinicopathologic characteristics of the 95 patients are summarized in Table 1. The patients were aged from 8 to 82 years (mean: 29.1 ± 15.9 years). Fifty-seven were male (mean: 30.9 ± 15.2 years, range: 9-82 years) and 38 were female (mean: 26.5 ± 16.4 years, range: 8-76 years). Sixty-six patients had SIT, 23 had MM, 3 had cecal malrotation, in two the anomaly was previously unnoted and in one case, the end of the appendix running along the anterior side of sacrum was found in the left side. According to localization of the symptoms, 59 patients presented with left and 14 with right lower quadrant pain, 7 with bilateral lower quadrant pain, 7 with left upper quadrant pain, 6 with peri-umbilical, and two presented with pelvic pain. With regard to the diagnosis, 49 patients were diagnosed with appendicitis during the pre-operative period, in 19 patients, the diagnosis was established intraoperatively and in 5 postoperatively; 14 patients were previously known to have SIT and/or MM. No information was available in eight patients. Of 95 patients included in this literature review, 13.6% of patients underwent laparoscopic surgery. Laparoscopic appendectomy was performed in eight cases[6,10,12,13,15,21,24,58], and was combined with cholecystectomy in two[3,41] and with ablation of endometriosis implants in one patient[7]. In the remaining two patients, the intervention was switched to laparotomy due to technical reasons[8,25].

DISCUSSION

There are two anatomic abnormalities which result in LSAA - the first being SI, and the second, less common abnormality, is MM[6,7]. MM is the term used to describe a spectrum of congenital positional anomalies of the intestine caused by nonrotation or incomplete rotation of the primitive loop around the axis of the superior mesenteric artery (SMA) during fetal life. Although about 80% of cases are diagnosed in patients younger than 1 mo, malrotation has also been reported in older children and adults[8]. The incidence of MM cited in the literature varies from 0.03% to 0.5% in live births[1,3,9,10]. SI is an uncommon condition, which is caused by a single autosomal recessive gene with incomplete penetrance and occurs in 1 per 5000 to 1 per 10000 live births[7]. This condition may be complete (SIT), when both thoracic and abdominal organs are transposed, or partial, when only one of those cavities is affected[1]. The incidence of SIT reported in the literature varies from 0.001% to 0.01% in the general population[12-14], whereas the incidence of acute appendicitis associated with SIT is reported to be between 0.016% and 0.024%[5,13,14].

According to published reports in the literature, LSAA occurs between the age of 8 and 63 years and is 1.5-fold more frequent in men than in women[3,13]. In our review, we determined the mean age of the patients as 29.3 ± 16.1 (range: 8-82) years and the male/female sex ratio as 3/2[1].

The differential diagnosis of left lower quadrant abdominal pain includes: diverticulitis, renal colic, ruptured ovarian cyst, Meckel's diverticulitis, epididymitis, incarcerated or strangulated hernia, bowel obstruction, regional enteritis, psoas abscess, and right- and left-sided appendicitis (LSAA)[1,4].

LSAA is a diagnostic dilemma, because the appendix is located in an abnormal position. The differential diagnosis of LSAA may not be promptly established in the emergency setting and is often delayed due to lack of uniformity in the clinical signs[11,58]. It is assumed that even though the viscera are transposed, the nervous system may not show the corresponding transposition, which may result in confusing symptoms and signs. In about 18.4%-31% of patients with SIT and MM, the pain caused by LSAA has been reported in the right lower quadrant[1,5,11-13]. In this literature review, it was observed that 14.7% of patients had pain localized in the right lower quadrant, which indicates the importance of accurate preoperative diagnosis in order to avoid incorrect incision.

The diagnosis of acute appendicitis in patients with SIT or MM can be based on physical examination, electrocardiogram, chest X-ray, barium studies, USG, CT scan and diagnostic laparoscopy[1,2].

Plain radiographs are usually not helpful for establishing the diagnosis of appendicitis. However, the detection of dextrocardia on chest X-ray and right-sided gastric bubble on abdominal plane X-ray is of considerable value in establishing the diagnosis of SIT. Barium enema with gastrografin can reveal MM or SIT, when there are difficulties in making the diagnosis of acute left lower quadrant pain[11]. Over the last two decades, there has been an increasing use of imaging modalities, such as USG and

CT, in the diagnosis of acute appendicitis. USG is widely used in cases of appendicitis, however, it has significant limitations: it is operator-dependent, and examination of the lower quadrant can be compromised in patients with large body habitus or by overlying bowel gas. The value of CT in the diagnosis of acute appendicitis has been well-documented, with a reported accuracy of 90%-98%[2,13]. USG and CT may also be helpful in the detection of SIT and MM. Of the patients included in this literature review, CT has been used in the diagnosis of 28 patients and USG in 22 patients since 2000[1,6,8,17-24,26,27].

After establishing the diagnosis of SIT or MM, the surgical options are the same as for normal patients[1]. According to the reviewed literature, we observed that many open and a few laparoscopic procedures have been performed[1,6,8,15]. Laparoscopic appendectomy was first carried out in 1998 by Contini *et al*[58] in a 34-year-old male patient with SIT. Since then, laparoscopic appendectomy has been performed in a total of 20 cases (12 with MM and 8 with SIT), of which two have undergone cholecystectomy at the same surgical session[3,6,7,10,12,13,15,21,24,41,58,65]. We believe that laparoscopy may be very useful both in establishing the differential diagnosis and in performing the definitive surgery[1].

As in patients with normally localized appendix, appendectomy specimens in LSAA should be sent for pathological evaluation. To our knowledge, in the literature, only two of 95 patients (59 male, 76 female), who underwent appendectomy due to LSAA, were pathologically diagnosed with malignancy. Ascendent hemicolectomy was performed in both patients after pathological evaluation, which revealed mucinous adenocarcinoma and mucinous cystadenocarcinoma[19,26].

In conclusion, LSAA should be considered in the differential diagnosis of young patients presenting with pain localized in the left lower quadrant. Chest X-ray, abdominal USG and CT provide quite useful information. Diagnostic laparoscopy is the gold standard in cases with complicated differential diagnosis.

COMMENTS

Background

Acute appendicitis is a surgical condition, which manifests itself as pain usually localized in the lower right quadrant and can be diagnosed easily due to its clinical findings. Besides the surgeon experience, blood tests, radiological investigations, and in some circumstances, diagnostic laparoscopic techniques, should be used in the diagnosis of atypically localized appendicitis.

Research frontiers

Left-sided appendix is a rarely seen condition, which is frequently associated with situs inversus totalis (SIT) and midgut malrotation (MM). In this study, the authors performed an overall evaluation of management approaches and diagnostic processes in cases of left-sided acute appendicitis (LSAA) related to SIT and MM published in the English language literature to date, and presented the experience.

Innovations and breakthroughs

This study is the largest literature screening on LSAA to date.

Applications

This brief literature review demonstrates the importance of radiologic assessment and diagnostic laparoscopy along with surgical experience in the differential diagnosis of LSAA.

Peer review

The authors described a case of left sided appendicitis in the setting of situs inversus and a corresponding literature review. Over all, this paper is well written, concise and information.

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BRIEF ARTICLE

Fresh frozen plasma transfusion does not affect outcomes following hepatic resection for hepatocellular carcinoma

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Abstract

AIM: To investigate whether fresh frozen plasma (FFP) transfusion affects outcomes following hepatic resection for hepatocellular carcinoma (HCC) in terms of liver function, postoperative complications and cancer prognosis.

METHODS: We retrospectively compared the incidence of postoperative complications between 204 patients who underwent hepatectomy for HCC with routine FFP transfusion in an early period (1983-1993, Group A) and 293 with necessity for FFP transfusion during a later period (1998-2006, Group B), and also between two subgroups of Group B [22 patients with FFP transfusion (Group B1) and 275 patients without FFP transfusion (Group B2)]. Additionally, only in limited patients in Group B1 and Group B2 with intraoperative blood loss $\geqslant$ 2000 mL (Group B1$_{\geqslant 2000\ mL}$ and Group B2$_{\geqslant 2000\ mL}$), postoperative complications, liver function tests, and cancer prognosis were compared.

RESULTS: No mortality was registered in Group B, compared to 8 patients (3.9%) of Group A. The incidence of morbidity in Group B2 [23.2% (64/275)] was not significantly different from Group B1 [40.9% (9/22)] and Group A [27.0% (55/204)]. The incidence of complications and postoperative liver function tests were comparable between Group B1$_{\geqslant 2000\ mL}$ *vs* Group B2$_{\geqslant 2000\ mL}$. Postoperative prognosis did not correlate with administration of FFP, but with tumor-related factors.

CONCLUSION: The outcome of hepatectomy for HCC is not influenced by FFP transfusion. We suggest FFP transfusion be abandoned in patients who undergo hepatectomy for HCC.

Key words: Fresh frozen plasma; Hepatocellular carcinoma; Surgery; Transfusion

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INTRODUCTION

Surgical resection is the established optimal treatment for hepatocellular carcinoma (HCC) associated with hepatitis B virus or hepatitis C virus infection. Since HCC usually develops in patients with liver cirrhosis, most such patients present with bleeding tendencies based on chronic liver dysfunction[1,2]. Accordingly, bleeding is a major problem in liver surgery for HCC, and it also affects postoperative mortality and morbidity[3-5].

Fresh frozen plasma (FFP) is human donor plasma, and contains near normal levels of many plasma proteins, including procoagulants and inhibitory components of the coagulation cascades, acute phase proteins, immunoglobulins and albumin. The clinical use of FFP has increased steadily over the last two decades in many countries[6-8]. Furthermore, in the surgical treatment of HCC, FFP has been frequently administered to supply coagulation factors, maintain serum albumin level and circulating blood volume, and prevent postoperative hepatic failure[9-12]. On the other hand, FFP transfusion is reported to induce adverse effects in some patients: transmission of infection, allergic reactions, hemolysis, anaphylaxis, and transfusion-related acute lung injury (TRALI)[13-15]. Moreover, some studies have reported a relationship between perioperative transfusion and postoperative HCC recurrence[16,17]. In addition to these adverse effects, the amount of FFP is limited because of its source from human donation. Therefore, appropriate use of FFP is needed in terms of application and volume, as stated in the guidelines of the Japanese Ministry of Health, Labour and Welfare[18]. Regarding surgery for HCC, recent advances in both surgical and anesthetic techniques that have led to a reduction in intraoperative blood loss, have resulted indirectly in a gradual decrease in the need for FFP perioperatively[19,20]. Considering the reduction in intraoperative blood loss and the aforementioned potential adverse effects of FFP transfusion, we believe there is no need for FFP in surgery for HCC. In order to discuss this need, we first should investigate whether FFP transfusion affects outcomes following hepatic resection for HCC.

In this study, we retrospectively investigate whether FFP transfusion affects outcomes following hepatic resection for HCC in terms of liver function, postoperative complications and cancer prognosis.

MATERIALS AND METHODS

Trends in transfusion

Until 1993, FFP was routinely administered to patients after hepatectomy for HCC at the Department of Surgery, Osaka University Hospital. In 1994, HCC patients began to donate their blood preoperatively for autologous blood transfusion during or after surgery. Between 1994 and 1997, the use of autologous blood transfusion and FFP transfusion was determined by the surgeon. However, in 1998, the use of autologous blood transfusion was implemented in our institution to cover all HCC patients with hemoglobin of ≥ 11.0 g/dL. FFP was administered only to patients with extensive bleeding intraoperatively and low levels of coagulation factors. After the publication of Guidelines by the Japanese Ministry of Health and Welfare, we adhered to these guidelines in the use of FFP[18].

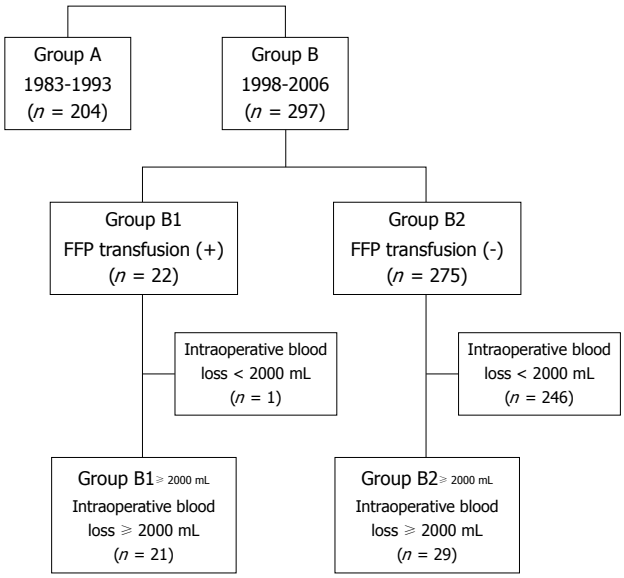

Figure 1 Distribution of the enrolled patients according to the clinical background of hepatectomy for hepatocellular carcinoma. FFP: Fresh frozen plasma.

Patients

Between 1998 and 2006, 297 patients underwent curative hepatic resection for HCC in our institution. In this study, we retrospectively compared the incidence of postoperative complications and postoperative cancer prognosis in the 297 patients with those of 204 patients with HCC who underwent curative hepatic resection with the routine use of FFP between 1983 and 1993. These 204 patients and 297 patients were categorized into Group A and Group B, respectively. The 297 patients of Group B were also divided into two groups depending on their history regarding perioperative FFP transfusion: 22 patients (7.4%) with FFP transfusion (Group B1) and 275 patients (92.6%) without FFP transfusion (Group B2). The distribution of patients enrolled in this study is illustrated accordingly in Figure 1. In patients of Group B1, FFP transfusion was performed either during the surgery or within 3 d after surgery. The median number of total units of transfused FFP was 10 (range, 4-40). In these groups, the need and validity of routine FFP transfusion were retrospectively evaluated based on the following postoperative complications and cancer prognosis.

Surgery and postoperative complications

In our institution, indication for hepatectomy for HCC is based on the value of indocyanine green retention rate at 15 min, and five factors included in the Child-Pugh classification: albumin, prothrombin time (PT), total bilirubin

Table 1 Comparison of perioperative characteristics between Group A (1983-1993) and Group B (1998-2006), and between Group B1 and Group B2 *n* (%)

	Group A (1983-1993) (*n* = 204)	Group B (1998-2006) (*n* = 297)	*P*-value	Group B		*P*-value
				Group B1 (*n* = 22)	Group B2 (*n* = 275)	
Age (yr)						
< 60	114 (55.9)	207 (69.7)	0.0015	9 (40.9)	81 (29.5)	NS
≥ 60	90 (44.1)	90 (30.3)		13 (59.1)	194 (70.5)	
Gender						
Male	178 (87.3)	235 (79.1)	0.0188	20 (90.9)	215 (78.2)	NS
Female	26 (12.7)	62 (20.9)		2 (9.1)	60 (21.8)	
Child-Pugh						
A	187 (91.7)	251 (84.5)	0.0176	17 (77.3)	234 (85.1)	NS
B	17 (8.3)	46 (15.5)		5 (22.7)	41 (14.9)	
Viral infection						
HBV (+)	40/201 (19.9)	56 (18.9)	NS	4 (18.2)	52 (18.9)	NS
HCV (+)	47/78 (60.3)	177 (59.6)	NS	11 (40.9)	166 (61.1)	NS
Surgical procedure						
≤ Hr1	171 (83.8)	241 (81.1)	NS	14 (63.6)	227 (82.5)	0.0436
≥ Hr2	33 (16.2)	56 (18.9)		8 (36.4)	48 (17.5)	
Intraoperative blood loss (mL)						
< 2000	133 (65.2)	247 (83.2)	< 0.0001	1 (4.5)	246 (89.5)	< 0.0001
≥ 2000	71 (34.8)	50 (16.8)		21 (95.5)	29 (10.5)	
Use of FFP						
(-)	0 (0)	275 (92.6)	< 0.0001			
(+)	204 (100.0)	22 (7.4)				
Mortality	8 (3.9)	0 (0)	0.0007	0 (0)	0 (0)	--
Morbidity	55 (27.0)	73 (24.6)	NS	9 (40.9)	64 (23.2)	NS

HBV: Hepatitis B virus; HCV: Hepatitis C virus; ≤ Hr1: Partial resection, subsegmentectomy, and segmentectomy of the liver; ≥ Hr2: Bisegmentectomy or more; FFP: Fresh frozen plasma; NS: Not significant.

(T-Bil), presence of ascites, and presence of encephalopathy. The selected surgical procedure was based on tumor location and predicted residual liver function, according to the classification system of the Liver Cancer Study Group of Japan[21]. The indication for surgery and selection of surgical procedure were not different between Group A and Group B. Death within 30 d after surgery was considered operative mortality. Morbidities were represented by the following complications that required additional treatment: cardiopulmonary complications, hepatic failure, bleeding, bile leakage, ascites and/or pleural effusion, ileus, and wound infection. PT and T-Bil [preoperative, postoperative day (POD) 1, 3, 5, 7] were used as representative markers of postoperative liver function.

Statistical analysis and ethical considerations

Differences between groups were assessed by the χ^2 test, Fisher's exact test or the Mann-Whitney *U* test. Survival rates were calculated according to the Kaplan and Meier method and compared using the log-rank test. Statistical analysis was performed using StatView (version 5.0, SAS Institute Inc., Cary, NC). A *P* value < 0.05 was considered statistically significant. The study was approved by the Human Ethics Review Committee of Osaka University Hospital and a signed consent form was obtained from each patient.

RESULTS

Table 1 lists the differences in perioperative characteristics between Group A and Group B. Patients classified as Child-Pugh A were significantly more common among Group A than Group B (*P* = 0.0176). Intraoperative blood loss in Group A was significantly greater than in Group B (*P* < 0.0001). While the postoperative mortality was 3.9% (8/204) in Group A, no mortality was recorded in Group B (*P* = 0.0007). The incidence of postoperative complications was 27.0% (55/204) in Group A and 24.6% (73/297) in Group B, and the incidence did not significantly differ between the two groups.

Various perioperative parameters were compared between Group B1 and Group B2 (Table 1). The preoperative factors were similar in the two groups. The incidence of hepatectomy equal to or more than Hr 2 was significantly higher in Group B1 than in Group B2 (*P* = 0.0436), and a significantly greater intraoperative blood loss was recorded in Group B1 than in Group B2 (*P* < 0.0001). There was no operative mortality in either of the two groups. The incidence of postoperative complications was 40.9% (9/22) in Group B1 and 23.2% (64/275) in Group B2, and the incidence did not significantly differ between the two groups. No adverse events related to FFP transfusion were found in Group B1. Postoperative complications and liver function were compared between Group B1 and Group B2 only in patients with intraoperative blood loss of ≥ 2000 mL (Group B1≥ 2000 mL: *n* = 21, Group B2≥ 2000 mL: *n* = 29). Comparison of clinical features of patients in these two groups is summarized in Table 2. There were no significant differences in the preoperative factors. Intraoperative blood loss and the frequency of administra-

Table 2 Comparison of perioperative characteristics between Group B1≥ 2000 mL and Group B2≥ 2000 mL *n* (%)

	Group B1≥ 2000 mL (*n* = 21)	Group B2≥ 2000 mL (*n* = 29)	*P*-value
Age (yr)			
< 60	9 (42.9)	11 (37.9)	NS
≥ 60	12 (57.1)	18 (62.1)	
Gender			
Male	20 (95.2)	23 (79.3)	NS
Female	1 (4.8)	6 (20.7)	
Child-Pugh			
A	17 (81.0)	21 (72.4)	NS
B	4 (19.0)	8 (27.6)	
Viral infection			
HBV (+)	5 (23.8)	3 (10.3)	NS
HCV (+)	9 (42.9)	19 (65.5)	NS
Maximum size of tumor(s) (cm)	8.3 ± 6.3	6.9 ± 4.6	NS
Intrahepatic metastasis			
(-)	9 (26.8)	17 (58.6)	NS
(+)	12 (63.2)	12 (41.4)	
Vascular involvement			
(-)	8 (38.1)	17 (58.6)	NS
(+)	13 (61.9)	12 (41.4)	
Operative time (min)	426 ± 154	391 ± 130	NS
Intraoperative blood loss (mL)	5364 ± 1651	2854 ± 1056	< 0.0001
Use of RCC			
(-)	3 (14.3)	19 (65.5)	0.0004
(+)	18 (85.7)	10 (34.5)	
Surgical procedure			
≤ Hr1	14 (66.7)	17 (58.6)	NS
≥ Hr2	7 (33.3)	12 (41.4)	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; ≤ Hr1: Partial resection, subsegmentectomy, and segmentectomy of the liver; ≥ Hr2: Bisegmentectomy or more; RCC: Red cell concentrate; NS: Not significant.

Table 3 Comparison of postoperative complications between Group B1≥ 2000 mL and Group B2≥ 2000 mL *n* (%)

	Group B1≥ 2000 mL (*n* = 21)	Group B2≥ 2000 mL (*n* = 29)	*P*-value
Mortality	0 (0)	0 (0)	--
Morbidity	9 (42.9)	9 (31.0)	NS
Cardiopulmonary	2 (9.5)	0 (0)	
Renal dysfunction	0 (0)	0 (0)	
Hepatic failure	0 (0)	0 (0)	
Bleeding	1 (4.8)	1 (3.4)	
Bile leakage	1 (4.8)	2 (6.9)	
Ascites and/or pleural effusion	0 (0)	3 (10.3)	
Ileus	2 (9.5)	0 (0)	
Wound infection	3 (14.3)	3 (10.3)	

NS: Not significant.

tion of red cell concentrates (RCC) in Group B1≥ 2000 mL were significantly more than those in Group B2≥ 2000 mL ($P < 0.0001$ and $P = 0.0004$, respectively). Operative mortality was not encountered in the two groups. The incidence of postoperative complications was 42.9% (9/21) in Group B1≥ 2000 mL and 31.0% (9/29) in Group B2≥ 2000 mL. Table 3 lists the types of complications. Neither postoperative hepatic failure nor postoperative bleeding occurred in the two groups.

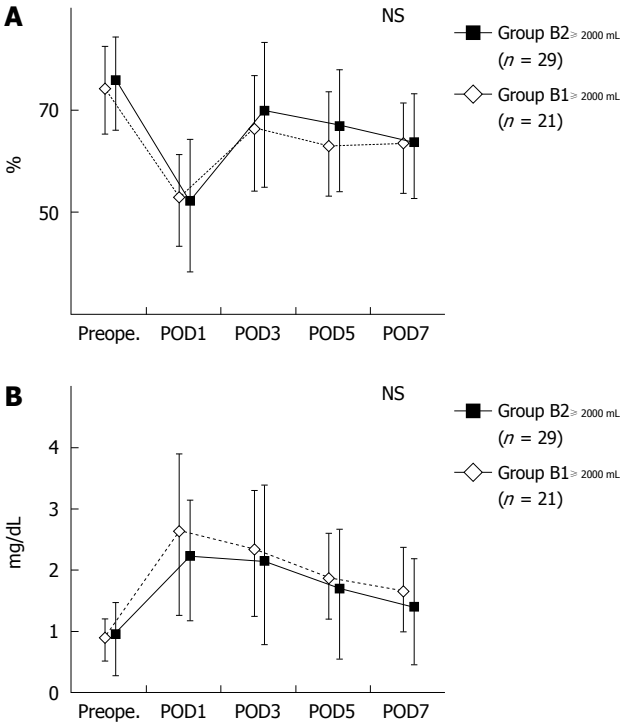

Figure 2 Perioperative changes in (A) serum prothrombin time, and (B) total bilirubin levels in Group B1≥ 2000 mL and Group B2≥ 2000 mL. NS: Not significant; Preope.: Preoperative; POD: Postoperative day.

Figure 2 demonstrates the perioperative changes in PT and T-Bil in patients with intraoperative blood loss of ≥ 2000 mL. The levels of PT and T-Bil were not significantly different between the two groups, irrespective of the POD.

Long-term postoperative outcomes, including disease-free survival (DFS) and overall survival (OS) after hepatic resection, were also examined in patients with intraoperative blood loss of ≥ 2000 mL (Table 4). Vascular invasion (absent/present), FFP transfusion (transfused/non-transfused), tumor size (< 5 cm/≥ 5 cm), RCC transfusion (transfused/non-transfused) were significant factors in univariate analysis of DFS among the clinicopathological factors tested ($P = 0.0101$, 0.0276, 0.0288, and 0.0343, respectively). Multivariate analysis for DFS using the four factors identified vascular invasion as the only significant independent factor ($P = 0.0299$). The DFS in Group B2≥ 2000 mL was significantly better than in Group B1≥ 2000 mL ($P = 0.0276$), though the factor was not significant on multivariate analysis (Figure 3A). Next, univariate analysis for OS using various clinicopathological factors demonstrated that vascular invasion (absent/present) and number of nodules (single/multiple) were significant factors ($P = 0.0024$ and $P = 0.0150$, respectively). Multivariate analysis for OS using the two factors, identified vascular invasion as the only significant independent factor ($P = 0.0185$). There was no significant difference in OS between Group B2≥ 2000 mL and Group B1≥ 2000 mL (P = not significant) (Figure 3B).

Table 4 Multivariate analysis of disease-free survival and overall survival after hepatectomy for hepatocellular carcinoma in patients with intraoperative blood loss of ≥ 2000 mL

	n	DFS			OS		
		OR	95% CI	*P*-value	OR	95% CI	*P*-value
Maximum size of tumor (cm)		1.553	0.755-3.191	NS			
≤ 5	16						
> 5	31						
Tumor number					2.280	0.874-5.591	NS
Single	24						
Multiple	26						
Vascular invasion		2.445	1.091-5.464	0.0299	3.203	1.216-8.439	0.0185
(-)	25						
(+)	25						
RCC transfusion		1.695	0.674-4.261	NS			
(-)	22						
(+)	28						
FFP transfusion		1.340	0.512-3.005	NS			
(-)	29						
(+)	21						

DFS: Disease-free survival; OS: Overall survival; OR: Odds ratio; 95% CI: 95% confidence interval; RCC: Red cell concentrate; FFP: Fresh frozen plasma; NS: Not significant.

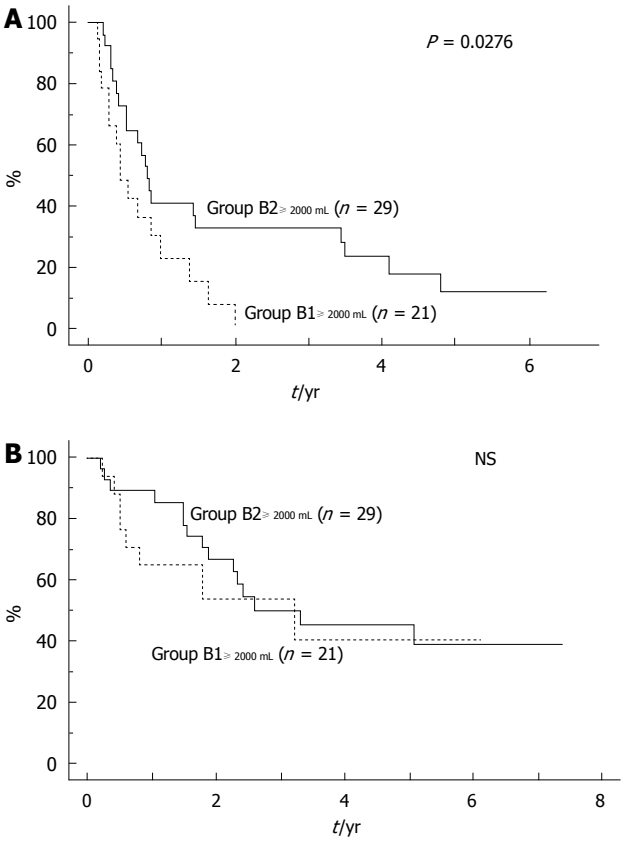

Figure 3 Disease-free survival (A) and overall survival (B) in Group B1≥ 2000 mL and Group B2≥ 2000 mL. NS: Not significant.

DISCUSSION

The present study was designed to investigate whether the outcomes of hepatectomy for HCC are influenced by FFP transfusion. Firstly, we compared the incidence of mortality and morbidity between Group A and Group B, indicating no significant difference in the incidence between the two groups. However, the comparison is considered to be difficult because of differences in the background of each period such as surgical and anesthetic techniques. For example, there were significant differences in liver function evaluated by Child-Pugh classification, and in intraoperative blood loss, between the two groups. Therefore, for more justified analysis, we next compared the outcomes between Group B1 and Group B2. The result showed the incidence of mortality and morbidity to be comparable between Group B1 and Group B2. However, since there were significant differences in the surgical procedure and intraoperative blood loss between the two groups, we also compared the postoperative complications between Group B1≥ 2000 mL *vs* Group B2≥ 2000 mL. The results showed equal rates of postoperative complications in the two groups. In particular, hepatic failure (prevention of which is one of the purposes of FFP administration) was not identified in the two groups. Postoperative residual liver function, represented by PT and T-Bil, was also equal in the two groups. Furthermore, the incidences of postoperative mortality and morbidity in Group B1 and Group B2 were similar to those reported in other studies[5,22-24]. For example, Imamura *et al*[5] reported the surgical result of 1056 hepatic resections including 532 HCC cases, with incidences of postoperative mortality and morbidity of 0% and 39.0%, respectively. However, they did not report the number of patients who received FFP. FFP was reported to be administered at a rate that exceeded the amount of blood loss by 10% to 20% during surgery; it substituted the amount of protein lost so as to maintain serum total protein level at 6.0 g/dL in that study. Based on our results and those of early studies, it cannot be concluded that FFP administration would contribute to the incidences of postoperative mortality and morbidity, including hepatic failure.

Although some previous studies have reported postoperative complications after hepatectomy for HCC, there are no reports comparing postoperative complications between patients with FFP transfusion and those without FFP transfusion. Martin *et al*[25] reported the use of FFP after hepatic resection and suggested criteria for FFP transfusion to deal with postoperative complications after treatment of liver metastasis from colorectal cancer, but not for HCC with liver cirrhosis. Accordingly, their criteria should not be necessarily generalized to the use of FFP in hepatectomy for HCC. Therefore, this study is the first report in which the incidence of postoperative complications in HCC patients was compared between patients who received FFP and those who did not receive FFP transfusion.

To date, FFP has been traditionally used at hepatectomy for the purpose of hemostatic effect by correction of deficiency of coagulation factors and maintenance of circulating blood volume by supplementation of albumin, which is mainly responsible for the colloid osmotic pressure of plasma; in addition to the aforementioned purpose of prevention of hepatic failure[6-8]. Firstly, with regard to the hemostatic effect, recent improvements in surgical techniques allow hepatectomy to be performed with minimal bleeding[19,20]. Moreover, coagulopathy requiring FFP transfusion is generally reported to occur at a PT value of more than 2.0 times the control, whereas the mean PT level of patients of Group B1≥ 2000 mL and Group B2≥ 2000 mL in the present study did not drop to the applicable level, even though it was measured after hepatectomy[26-28]. Furthermore, the incidence of postoperative bleeding was low and was not different in the two groups. Taking these results into consideration, routine administration of FFP is not necessary in terms of the hemostatic effect. Secondly, the maintenance of appropriate circulating blood volume is important in order to prevent certain complications such as pulmonary edema and prerenal type of renal dysfunction. However, albumin products, which can be administered safely compared to FFP, can be substitutes for FFP in terms of maintenance of circulating blood volume. In fact, albumin products were administered perioperatively instead of FFP in this study, especially in the non-transfused group, and the incidence of these complications was not different between Group B1≥ 2000 mL and Group B2≥ 2000 mL. In this context, routine FFP administration is also suggested not to be necessary in terms of maintenance of the circulating blood volume. Thus, we suggest that the routine administration of FFP for the purpose of prevention of hepatic failure, hemostatic effect, and maintenance of circulating blood volume is not necessary.

Many adverse effects related to FFP transfusion have been identified, such as infection, allergic reactions, hemolysis, anaphylaxis, and TRALI[13-15]. In particular, TRALI, which is a rare and serious complication characterized by sudden onset of respiratory distress due to noncardiogenic pulmonary edema during or following transfusion, can be life-threatening. Fortunately, none of these transfusion-related complications occurred in our patients. However, since some of the reported adverse events can be life-threatening, one should refrain from inappropriate use of FFP.

Since an initial report by Foster *et al*[29] about survival advantages in patients undergoing colectomy for colon cancer, several other reports have shown that perioperative homologous blood transfusion to be an independent prognostic factor in many kinds of cancers[16,17,30-33]. However, a few suggested that homologous blood transfusion has no significant effect on the prognosis of cancer patients[34,35]. Thus, the association between transfusion and postoperative prognosis is still under debate. In the present study, postoperative prognosis did not correlate with FFP administration, but rather with tumor-related factors. Although the result was not powerful evidence to resolve the controversy, we can at least confirm that FFP administration does not improve prognosis of patients undergoing hepatectomy for HCC.

In fact, the guidelines of the Japanese Ministry of Health, Labour and Welfare state that administration of FFP should be limited only to supplement coagulation factors in those patients with a PT of more than 2.0 times normal or coagulation factor activity of ≤ 30%, and that the use of FFP for supplementation of circulation blood volume is inappropriate[18]. The guidelines do not mention administration of FFP for the prevention of hepatic failure. Thus, our suggestion is to obey the guidelines. Recently, Kaibori *et al*[36] reported the clinical value of FFP in surgery for HCC. They suggested that FFP transfusion was useful and recommended on the grounds of the results obtained from their analysis that the incidence of postoperative complications in patients with FFP transfusions was lower than that of patients with FFP and RCC transfusions, and was equal to that of non-transfused patients; long-term survival in patients with FFP transfusions was almost equal to that in non-transfused patients. However, their suggestion is perceived as groundless for the following reasons. To begin with, although there were some significant differences in many factors such as liver function and tumor progression among the groups in their study, they simply suggested that the difference in postoperative complications and long-term outcome resulted from the RCC and FFP transfusions. Secondly, since details of postoperative complications were not shown, especially for hepatic failure, postoperative bleeding, pulmonary edema and renal dysfunction, the examination of correlations between complications and FFP transfusions was insufficient. In addition, their suggestion completely ignored the recent guidelines of Japan.

The present analysis did not include HCC patients who underwent liver transplantation for treatment of liver cirrhosis. Therefore, the result of this study is not applicable to liver transplantation surgery. Considering that transfusion is performed for concomitant liver dysfunction at almost all liver transplantation surgery, it seems to be still too early to discuss the necessity of transfusion in such surgery.

In summary, FFP transfusion did not affect outcomes following hepatic resection for HCC in terms of liver function, postoperative complications and cancer prognosis. Considering the previously reported FFP transfusion-related adverse effects in addition to the results of the present study, we suggest that FFP transfusion be abandoned in patients who undergo hepatectomy for HCC.

COMMENTS

Background

Fresh frozen plasma (FFP) has been frequently administered in the surgical treatment for hepatocellular carcinoma (HCC). Today, appropriate use of FFP is needed in terms of application and FFP transfusion-related potential adverse events. However, to our knowledge, there have been few reports investigating whether FFP transfusion affects outcomes following hepatic resection for HCC or any discussion of the need for FFP in surgery for HCC.

Research frontiers

The incidence of mortality and morbidity, postoperative liver function, and postoperative cancer prognosis were comparable between patients with intraoperative blood loss ⩾ 2000 mL who had FFP transfusion and who did not have FFP transfusion.

Innovations and breakthroughs

This study showed that FFP transfusion did not affect outcomes following hepatic resection for HCC in terms of liver function, postoperative complications and cancer prognosis.

Applications

Considering the results of the present study, there is a suggestion that FFP transfusion should be abandoned in patients who undergo hepatectomy for HCC.

Peer review

The manuscript is a well-written paper that is adequately discussed with a reasonable number of literature references. Moreover, the topic is a current and popular one. Conclusions are well supplied by the results and literature.

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BRIEF ARTICLE

MRI of magnetically labeled mesenchymal stem cells in hepatic failure model

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Abstract

AIM: To track intravascularly transplanted mesenchymal stem cells (MSCs) labeled with superparamagnetic iron oxide (SPIO) by using magnetic resonance imaging (MRI) in an experimental rabbit model of hepatic failure.

METHODS: Human MSCs labeled with FDA-approved SPIO particles (Feridex) were transplanted *via* the mesenteric vein into rabbits (n = 16) with carbon tetrachloride-induced hepatic failure. Magnetic resonance (MR) examinations were performed with a 3.0 T clinical scanner immediately before and 2 h and 1, 3, and 7 d after transplantation. Signal intensity (SI) changes on T2*-weighted MRI were measured, and correlation between MR findings and histomorphologic findings was also investigated.

RESULTS: SI on T2*-weighted MRI decreased significantly in the liver 2 h after injection of human MSCs and returned gradually to the levels found before injection in 7 d. Changes in SI in the liver at 2 h, 1, 3, and 7 d were 41.87% ± 9.63%, 10.42% ± 4.3%, 5.12% ± 1.9%, 3.75% ± 1.2%, respectively (P < 0.001). Histologic analyses confirmed the presence of MSCs in the liver, localized mainly in the sinusoids in early period (2 h and 1 d) and concentrated to the border zone in late period (3 and 7 d). The number of iron-positive cells in the liver at 2 h and on 1, 3 and 7 d after transplantation was 29.2 ± 4.8, 10.1 ± 3.7, 6.7 ± 2.2, and 5.8 ± 2.1, respectively (P = 0.013).

CONCLUSION: Intravascularly injected SPIO-labeled MSCs in an experimental rabbit model of hepatic failure can be detected and followed with MRI.

Key words: Stem cell; Liver failure; Magnetic resonance; Nanoparticle; Transplantation

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INTRODUCTION

As a potential treatment, liver cell transplantation provides an effective strategy for the treatment of liver failure[1]. Transdifferentiation of stem cells of various types into functional hepatocytes has been demonstrated, including embryonic stem cells, hepatoblasts, hepatic oval cells, pancreatic progenitor cells, bone marrow hematopoietic stem cells, and mesenchymal stem cells (MSCs)[2-7]. The use of MSCs as a potent potential source for hepatocytic transdifferentiation has been tried[8,9] and bone marrow-derived MSCs protected against carbon tetrachloride-induced liver fibrosis in rats[10].

In the past several years, a great deal of research has been focused on stem cell therapy. However, there remain many important issues to be addressed regarding cell therapy, and noninvasive and repeated monitoring of *in vivo* transplanted stem cells is an important topic in these days. Therefore, more recent research activities have focused on *in vivo* real-time tracking and detecting the fate of transplanted stem cells by using appropriate imaging technologies[11,12].

The use of magnetic resonance imaging (MRI) is well suited to evaluate the ability of cells to migrate and engraft to target organs, as MRI can provide a detailed anatomy of target organs with excellent spatial resolution. Paramagnetic or modified dextran-coated superparamagnetic iron oxide (SPIO) contrast agents have been used to label cells, allowing investigators to monitor cellular migration using MRI[12,13]. However, few studies have been undertaken to determine the feasibility of the use of *in vivo* MRI of cell therapy in models of hepatic failure. The aim of the present study is to assess *in vivo* MRI with the use of a clinical 3-Tesla MRI unit for the depiction of SPIO-labeled MSCs in a rabbit model of hepatic failure.

MATERIALS AND METHODS

Cell culture and labeling

The method of cell culture and labeling was previously reported[14] and is only briefly described here. Human MSCs (Bio-Whittaker, Walkersville, MD) were grown in mesenchymal stem cell basal medium (MSCBM, Bio-Whittaker) at 37℃ and in a 95% air, 5% CO_2 atmosphere. Human MSCs were cocultured in MSCBM containing FDA-approved SPIO particles (Feridex; Berlex, Wayne, NJ). The iron concentrations of the SPIO preparations were 125 μg/mL. Poly-L-Lysine (PLL) (Sigma-Aldrich, St. Louis, MO) was used as a transfection agent.

Animal models

All animal work was conducted in accordance with the guidelines provided by the Institutional Animal Control and Utilization Committee. The experiments were performed with 24 New Zealand white rabbits weighing 2.5-3.2 kg. The average age of rabbits was 10 wk. The rabbits were allowed food and water *ad libitum* and were kept in 44 cm × 68 cm × 39 cm sized cage with 22 ± 2℃ of temperature and 40%-60% of humidity.

Hepatic fibrosis was induced by intragastric administration of carbon tetrachloride (CCl_4) twice a week for 2 wk with 0.1 mL/kg in olive oil (at a 1:1 ratio). One day after the fourth administration of CCl_4, rabbits were anesthetized with intramuscular injection of 25-50 mg/kg ketamine hydrochloride (Yuhan Yanghang) and 10-20 mg/kg 2% xylazine hydrochloride (Bayer). After the abdomens of the rabbits were opened to expose the mesenteric vein, 1×10^6 SPIO-labeled human MSCs were slowly injected into the mesenteric vein in the experimental group (16 rabbits). One milliliter normal saline was injected into the mesenteric vein in control group (8 rabbits).

MRI

In all 24 rabbits MRI was performed with a 3.0 T MRI scanner (Signa Excite; GE Healthcare, Milwaukee, WI) with a knee coil. MRI of the rabbit liver was carried out immediately before, and 2 h, and 1, 3, and 7 d after injection of these stem cells. Transverse T2*-weighted gradient-echo (repetition time msec/echo time msec, 600/20; flip angle, 30°; section thickness, 3 mm; FOV, 18 cm; matrix, 256 × 160; number of signals acquired, two) sequences were employed.

Changes in signal intensity (SI) were characterized by use of region of interest (ROI) analysis on a well-centered slice with an area of 1 mm^2. A minimum of 15 pixels was required per region as displayed on a picture archiving and communication system (PACS) workstation (Maroview; Marotech). The value of the ROI was determined as the mean ± SD after an estimated three times, and was normalized to that of back muscle[15]. SI changes in each rabbit were calculated according to the use of the following formula: [SI (pre) - SI (post)]/SI (pre) × 100; SI (pre) and SI (post) are the normalized SI values as compared to back muscle before and after labeled cell injection.

Histological analysis

Two hours, and 1, 3, and 7 d after MRI, 4 rabbits each time in the experimental group and 2 rabbits each time in the control group were sacrificed for histological examination. Rabbit liver tissue blocks were fixed in 4% paraformaldehyde and were processed for paraffin embedding. Microsections (4 μm) were prepared with a microtome and were used for Prussian blue staining to detect labeled iron particles in the cells. An experienced board-certified pathologist calculated the number of Prussian blue stain positive cells per high power field (HPF) in each group. Prussian blue stain positive cells were counted in at least three HPFs per section and a minimum of six sections were examined.

Statistical analysis

The Kruskall-Wallis test was used to evaluate differences in SI changes and Prussian blue stain positive cells. *P* value of less than 0.05 was considered to indicate a statistical significance, and statistical computer software (SPSS 12.0; SPSS, Chicago, Ill) was used.

RESULTS

In the experimental group (n = 16), axial T2*-weighted

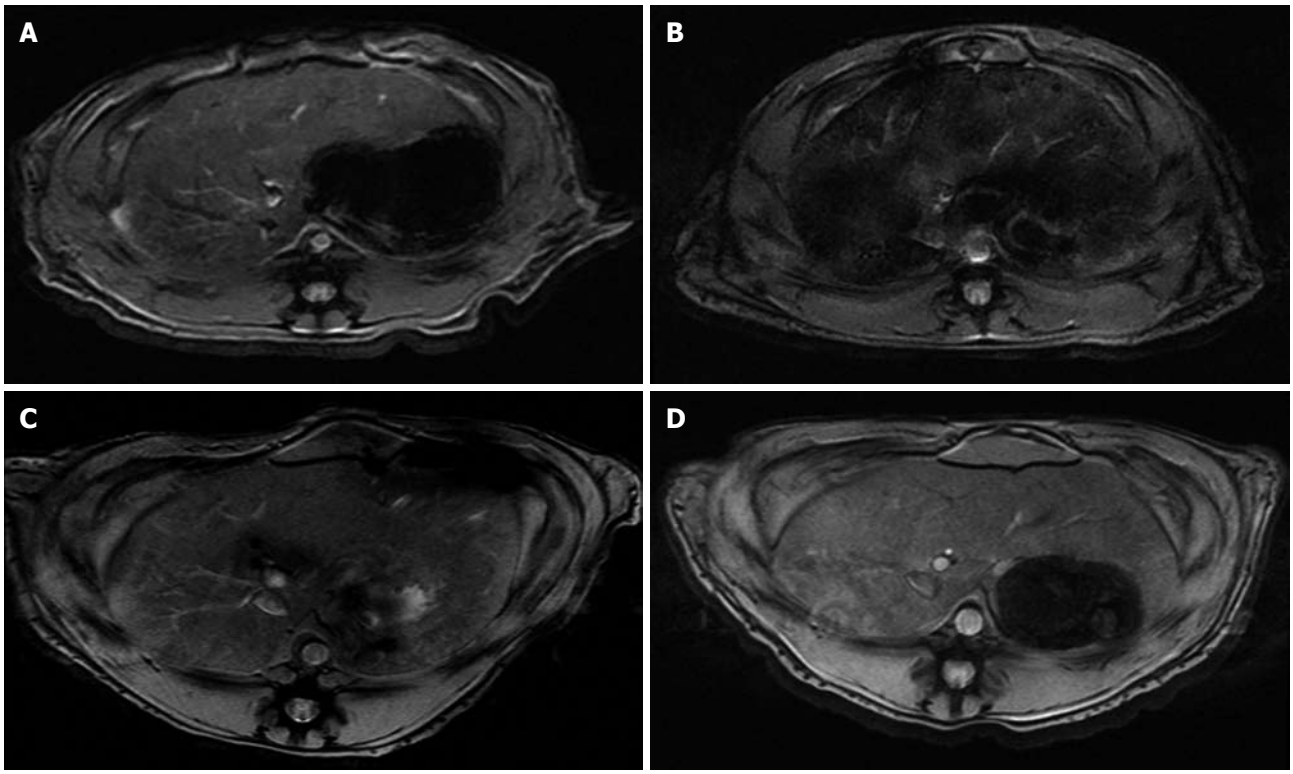

Figure 1 T2*-weighted gradient-echo magnetic resonance images show signal intensity changes of the liver after injecting superparamagnetic iron oxide-labeled human mesenchymal stem cells immediately before (A), 2 h (B) and 1 d (C), 7 d (D) after injection. Changes in signal intensity in the liver normalized to that of back muscle at 2 h, 1 d, and 7 d were 41.87% ± 9.63%, 10.42% ± 4.3%, and 3.75% ± 1.2%, respectively ($P < 0.001$). Note a significant signal drop of the liver at 2 h after injecting superparamagnetic iron oxide-labeled mesenchymal stem cells and gradual return of signal intensity compared with signal intensity before transplantation.

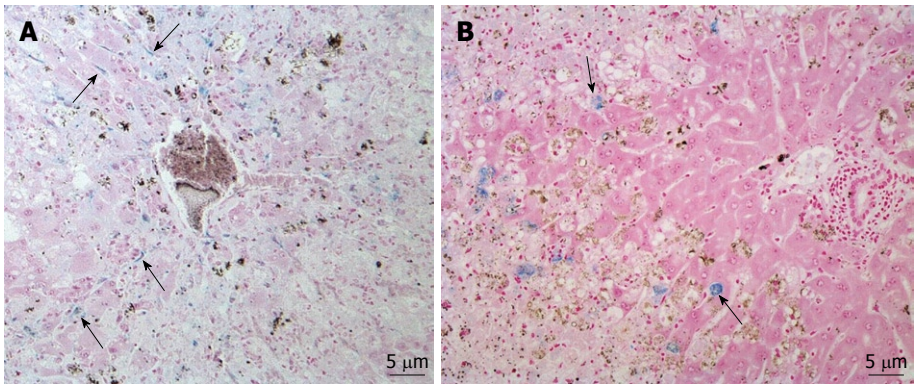

Figure 2 Photomicrographs of rabbit liver obtained after superparamagnetic iron oxide-labeled human mesenchymal stem cells injection *via* the mesenteric vein. A: Section obtained on day 1 shows distributions of Prussian blue stain-positive mesenchymal stem cells (MSCs) (arrows) in the liver parenchyma along the sinusoids; B: Section obtained on day 7 shows the localization of superparamagnetic iron oxide-labeled MSCs (arrows) in the border zone between normal liver parenchyma and hepatic injury areas.

images of the liver demonstrated a significant signal loss of fine granular signal voids 2 h after intravascular administration of SPIO-labeled cells in comparison to baseline (Figure 1). The change in SI of the liver on T2*-weighted images peaked at 2 h after injection and subsequently declined. Changes in SI in the liver at 2 h, 1, 3 and 7 d were 41.87% ± 9.63%, 10.42% ± 4.3%, 5.12% ± 1.9%, 3.75% ± 1.2%, respectively ($P < 0.001$). In the control group ($n = 8$), no overt signal changes of the liver were observed.

CCl4 treatment of rabbits induced typical histopathological changes indicative of persistent damage and fibrotic changes in the liver. Prussian blue staining studies showed that the iron-containing cells were mainly distributed in the portal triad regions throughout the liver 2 h and 1 d after injection of human MSCs. These cells showed amorphous shapes with positive iron staining in their cytoplasms and tended to localize along the sinusoids (Figure 2A). However, the iron-positive cells in the experimental group mainly appeared in the border zone between normal liver parenchyma and hepatic injury areas 3 and 7 d after injection (Figure 2B). The number of iron-positive cells in the experimental group at 2 h and on 1,

3 and 7 d after injection was 29.2 ± 4.8, 10.1 ± 3.7, 6.7 ± 2.2, and 5.8 ± 2.1, respectively (P = 0.013). In the control group, Prussian blue positively stained cells were absent from the liver.

DISCUSSION

It is widely accepted that efficient monitoring of the distribution, migration, and differentiation of transplanted stem cells holds the key to the development of effective methods in stem cell therapy[16]. Because of its high resolution and sensitivity, MRI has been regarded as a method of first choice compared to other imaging modalities. MRI can be broadly defined as a non-invasive and repetitive modality of imaging for targeted cells and cellular processes[17], which entails proper labeling of cells with appropriate magnetic resonance (MR) contrast agents.

In most reports that have described MRI of grafted stem cells, local implantation has been used as the route for cell grafting into the rat brain and spine[18,19]. After implantation, these cells are concentrated in a localized area rather than dispersed throughout the target organ, as occurs after intravascular injection. These locally implanted stem cells were observed to migrate only at a rate of a few millimeters a week[18-20]. This stereotactic method can hardly be applied in a hepatic failure model because liver damage often has a widespread distribution. Thus, we injected MSCs into the mesenteric vein, as a large number of MSCs could be directly transferred to the whole liver.

Our results and others[21] demonstrate that MSCs are particularly present along the sinusoids where the cells can be mechanically trapped after injection into the portal vein. Recently, the process of migration of stem cells from the spleen to the liver in rats was tracked *in vivo* by labeling stem cells with superparamagnetic particles[12], which showed that the majority of iron-positive cells appeared in the liver at 3 h of post-transplantation. This is similar to our findings. The results together suggest that direct intravascular transplantation is an efficient way of delivering MSCs to the liver.

The histological data obtained in the present study showed that Prussian blue stain-positive cells were found mainly in the border zone between normal liver parenchyma and the hepatic injury areas 3 and 7 d after injection. The exact reason why MSCs were concentrated in the border zone in liver damage is not known. This phenomenon may be caused by secreted cytokines such as hepatocyte growth factor which is secreted by mesenchymal cells and has a major role in adult organ regeneration and in wound healing[22]. We speculate MSCs might contribute the healing process of liver damage.

SI on T2*-weighted images decreased substantially in the liver 2 h after injection of MSCs and gradually returned to the levels found before injection. The gradual increase of SI in the liver may be the result of mobilization of labeled cells out of the liver, dilution of intracellular iron as a result of cell division, or cell death resulting from immune rejection[12]. In our study, SI decreased more quickly than that reported in other study[12] and this was probably due to the use of human MSCs in our study that induces more potent immune rejection than previous study.

There are some limitations to this study. First, we used only commercially available human MSCs, and not rabbit MSCs, because we focused on the validity of MRI for the depiction of SPIO-labeled MSCs in hepatic failure model rather than to access recovery of hepatic function. Second, we did not perform immunohistochemical analysis on human MSCs under the conditions that we tested with the rabbit model, and therefore could not confirm whether Prussian blue stained positive cells were associated with SPIO-labeled MSCs *in vivo*.

In conclusion, SPIO-labeled MSCs could be detected *in vivo* by the use of a clinically available 3-Tesla MR unit after intravascular injection in a rabbit model of hepatic failure.

COMMENTS

Background

Stem cell transplantation provides an effective strategy for the treatment of liver failure. Noninvasive and repeated monitoring of *in vivo* transplanted stem cells is one of important topics in these days.

Research frontiers

In vivo real-time tracking and detecting the fate of transplanted stem cells by magnetic resonance imaging (MRI) would be a good way to evaluate the ability of cells to migrate and engraft to target organs. However, few studies have been undertaken to determine the feasibility of the use of *in vivo* MRI of cell therapy in models of hepatic failure.

Innovations and breakthroughs

This study showed the possibility of stem cell tracking by the use of a clinically available 3-Tesla magnetic resonance unit.

Applications

In vivo real-time tracking transplanted stem cells by MRI might facilitate clinical use of stem cell.

Terminology

Mesenchymal stem cells: multipotent stem cells that can differentiate into a variety of cell types including osteoblasts (bone cells), chondrocytes (cartilage cells) and adipocytes (fat cells).

Peer review

It is a well-written but moderately designed study, with interesting and important scientific merit.

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BRIEF ARTICLE

Application of double-balloon enteroscopy in jejunal diverticular bleeding

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Abstract

AIM: To evaluate the efficacy of endoscopic diagnosis and therapy for jejunal diverticular bleeding.

METHODS: From January 2004 to September 2009, 154 patients underwent double-balloon enteroscopy (DBE) for obscure gastrointestinal bleeding. Ten consecutive patients with jejunal diverticula (5 males and 5 females) at the age of 68.7 ± 2.1 years (range 19-95 years) at Chang Gung Memorial Hospital, Academic Tertiary Referral Center, were enrolled in this study.

RESULTS: Of the 10 patients, 5 had melena, 2 had hematochezia, 2 had both melena and hematochezia, 1 had anemia and dizziness. DBE revealed ulcers with stigmata of recent hemorrhage in 6 patients treated by injection of epinephrine diluted at 1:10 000, Dieulafoy-like lesions in 4 patients treated by deploying hemoclips on the vessels, colonic diverticula in 2 patients, and duodenal diverticula in 3 patients, respectively. Of the 2 patients who underwent surgical intervention, 1 had a large diverticulum and was referred by the surgeon for DBE, 1 received endoscopic therapy but failed due to massive bleeding. One patient had a second DBE for recurrent hemorrhage 7 mo later, which was successfully treated with a repeat endoscopy. The mean follow-up time of patients was 14.7 ± 7.8 mo.

CONCLUSION: DBE is a safe and effective treatment modality for jejunal diverticular bleeding.

Key words: Double-balloon enteroscopy; Jejunal diverticular bleeding; Obscure gastrointestinal bleeding

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INTRODUCTION

Jejunal diverticulum, first described by Sir Astley Cooper in 1807, is a rare lesion of the small intestine seen in 2%-2.3% of small-bowel contrast studies[1,2] and 1.3%-4.6% of autopsy studies[3,4]. Most jejunal diverticula are asymptomatic, but they are usually complicated by diverticulitis with or without bowel perforation, intestinal obstruction, and bleeding, once they become symptomatic[5,6]. Massive gastrointestinal bleeding secondary to jejunal diverticulosis

is extremely rare, and only less than 50 cases have been reported[7]. Most of them present with obscure gastrointestinal bleeding.

In the past, jejunal diverticular bleeding was surgically treated[8], with associated operative morbidity and mortality, as well as postoperative ileus[9]. Double-balloon enteroscopy (DBE), a new technique developed by Yamamoto *et al*[10], using the push-and-pull method, enables optimal to-and-fro observation as well as therapeutic intervention[11]. Herein, we describe our clinical experience with this new technique in the diagnosis and treatment of jejunal diverticular bleeding at one academic tertiary-referral center in Taiwan.

MATERIALS AND METHODS

Patients

From January 2004 to September 2009, 154 patients with obscure gastrointestinal bleeding were referred for DBE at our tertiary referral center. Ten (6.5%) consecutive patients with jejunal diverticular bleeding (5 males and 5 females) at the age of 68.7 ± 2.1 years (range 19-95 years) at Chang Gung Memorial Hospital, Academic Tertiary Referral Center, were enrolled in this study. Obscure gastrointestinal bleeding is defined as the absence of an identified bleeding source after standard endoscopic evaluation[12,13]. The demographics of these patients are shown in Table 1. Of the 10 patients, five had melena, two had hematochezia, two had both melena and hematochezia, and one had microcytic anemia and dizziness.

Informed consent was obtained each patient prior to the endoscopic procedure.

DBE system and technique

The DBE system (Fujinon Inc., Japan) developed by Yamamoto *et al*[10] was used. A Fujinon EN-450 T5-type enteroscope with a 2.8-mm working channel and a 9.4-mm outer diameter was used, permitting easier and greater instrument access where necessary. Two soft latex balloons were fitted at tip of the enteroscope and overtube. An air pump controller (Fujinon PB-10) was used to measure and control the pressure in both balloons. Insertion route was selected based on the information obtained from pre-DBE. Peroral route examination was performed first, in the event of insufficient localization clues, because of the direction of peristalsis in the small intestine, which could thus reduce interference from blood in the small intestine. If no bleeder was identified, tattooing with pure carbon was performed at the most distal point during the examination. A second examination was performed a few days later. No special preparation was needed for the peroral examination other than an 8-12 h fasting. For the perianal examination, bowel preparation was performed as in colonoscopy. Mild sedation was accomplished *via* intravenous injection with meperidine (0.5-1.0 mg/kg) and midazolam (0.05 mg/kg). An Olympus rotary reusable delivery system, HX-110UR (2300 mm in length) with an EZ clip, was used for Dieulafoy-like lesions, and local injection with epinephrine diluted at 1:10 000 was applied to lesions with stigmata of recent hemorrhage (SRH).

RESULTS

Three patients were treated with non-steroidal anti-inflammatory drugs, one was treated with coumadin, and one was treated with clopidogrel. Esophagogastroduodenoscopy, colonoscopy or DBE showed colonic diverticula in two patients and duodenal diverticula in three patients, respectively. Other imaging studies including computed tomography (CT), barium study, angiography, and capsule enteroscopy (CE) were performed before DBE (Table 1). Five patients underwent CT scan with no positive CT finding found in any patient. Four patients underwent CE with diverticular bleeding detected in one patient. Eight patients underwent barium study and the results are shown in Table 1. Three patients underwent angiography with no positive finding observed in any patient.

All the 10 patients underwent peroral DBE, which was chosen according to the presenting clinical symptoms and signs, estimation of bleeding sites, and the results of previous studies.

Six patients (Table 2) had ulcers with SRH on the margin of diverticulum, which were treated by injection of 1-3 mL epinephrine diluted at 1:10 000 (Figure 1). Four patients had Dieulafoy-like lesions on the fold of diverticulum, which were treated by deploying hemoclips (Figure 2) on the vessels. The lesions were roughly estimated in the proximal to middle jejunum.

Of the two patients who underwent further surgical intervention, one had a large diverticulum (2.3 cm × 1.5 cm × 1.2 cm in size) and was referred by the surgeon for DBE, one experienced failure of endoscopic therapy due to massive bleeding. One patient underwent a second DBE for recurrent hemorrhage 7 mo later, and was successfully treated with a repeat endoscopy. The mean follow-up time of patients was 14.7 ± 7.8 mo. Occult blood testing was negative in stools of these patients, and no significant decrease was observed in hemoglobin during the follow-up period.

DISCUSSION

Gastrointestinal bleeding is frequently encountered by the endoscopist in his/her daily medical practice. Ninety-five percent of the bleeders can be identified by conventional upper endoscopy or colonoscopy. However, 5% of bleeders cannot be found even on repeat conventional upper endoscopy or colonoscopy. This phenomenon is known as obscure gastrointestinal bleeding, and can be further divided into frank bleeding (overt bleeding) and iron-deficiency anemia with guaiac-positive stools (occult bleeding)[12,13]. The bleeding site of most obscure gastrointestinal bleeders is in the small intestine. Lesions in potential bleeders can be further classified into 3 types: vascular lesions, neoplastic lesions, or inflammatory lesions, including diverticula[14].

Table 1 Demographics of 10 patients with jejunal diverticular bleeding

No.	Age (yr)/sex	Symptoms	Hb (g/dL)	Drugs	CT	CE	Barium	Angiography
1	64/M	Melena and hematochezia	8.9	-	Negative	-	Diverticulosis	-
2	61/F	Melena and hematochezia	9.8	NSAIDs -		-	Diverticula	-
3	80/M	Melena	11	NSAIDs	Negative	-	Diverticula	Negative
4	95/F	Melena	11.2	Coumadin	Negative	r/o right colonic bleeding	Negative	-
5	18/M	Dizziness	10	-	-	-	r/o P-J syndrome	-
6	83/M	Hematochezia	11.1	NSAIDs	-	-	Diverticula	-
7	81/F	Hematochezia	10.9	-	-	Diverticular bleeding	Diverticula	Negative
8	73/M	Melena	10.7	-	-	-	-	-
9	58/F	Melena	10.3	Clopidogrel	Negative	Diverticular	-	-
10	74/F	Melena	9.6	-	Negative	Diverticular	diverticular	Negative

CT: Computed tomography; CE: Capsule enteroscopy; NSAID: Non-steroidal anti-inflammatory drug; P-J: Peutz-Jeghers.

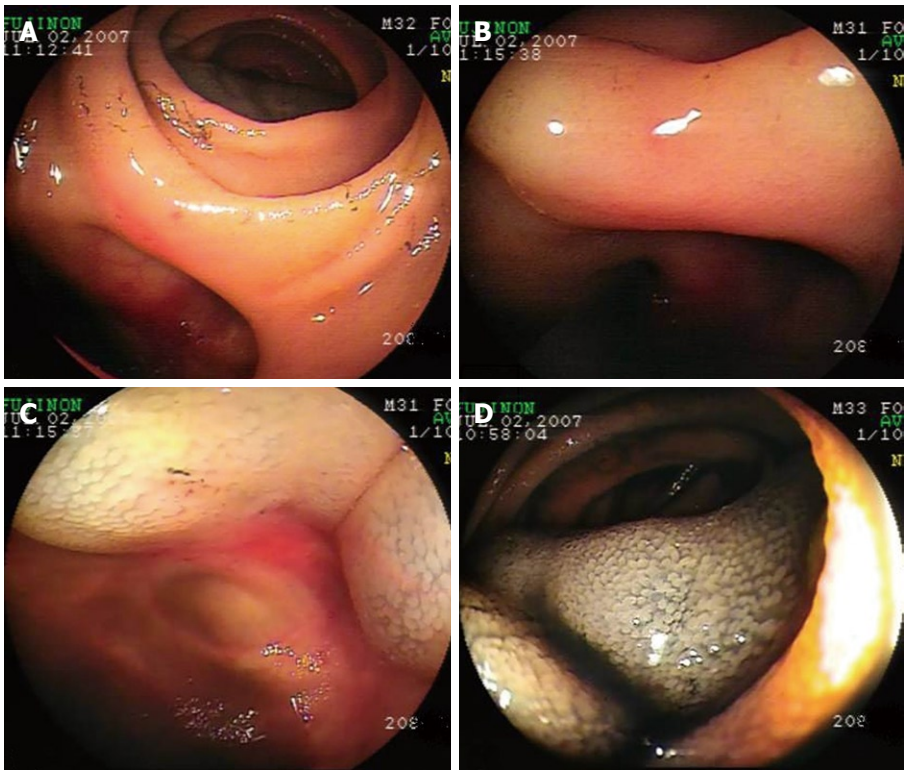

Figure 1 Jejunal diverticular bleeding treated by double-balloon enteroscopy local injection. A: A shallow ulcer with stigmata of recent hemorrhage on the diverticular margin; B, C: Local injection with epinephrine diluted at 1:10 000; D: Tattooing with pure carbon on the distal side of the diverticulum for marking.

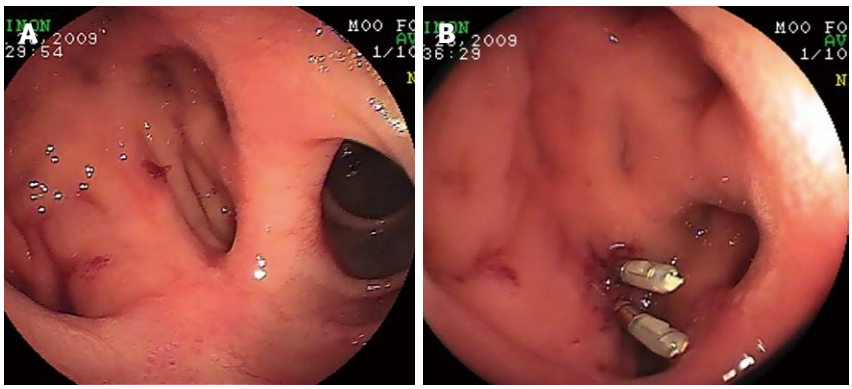

Figure 2 A Dieulafoy-like lesion observed in diverticular fold (A), which was managed by deploying two hemoclips on the vessel (B).

Table 2 Enteroscopy and double-balloon enteroscopy findings

No.	Route	Findings	Management	Follow-up timed (mo)	Outcome
1	Per-oral	Ulcer with SRH on the margin	Injection therapy	16	Recurrent bleeding after 7 mo, second DBE therapy performed
2	Per-oral	Ulcer with SRH on the margin	Injection therapy	14	Surgical intervention for large diverticulum
3	Per-oral	Dieulafoy-like lesion on the fold	Hemoclipping	9	Surgical intervention for failure of endoscopic treatment
4	Per-oral	Ulcer with SRH on the margin	Injection therapy	19	No recurrence
5	Per-oral	Ulcer with SRH on the margin	Injection therapy	22	No recurrence
6	Per-oral	Ulcer with SRH on the margin	Injection therapy	9	No recurrence
7	Per-oral	Dieulafoy-like lesion on the fold	Hemoclipping	8	No recurrence
8	Per-oral	Dieulafoy-like lesion on the fold	Injection therapy and hemoclipping	9	No recurrence
9	Per-oral	Ulcer with SRH on the margin	Injection therapy	32	No recurrence
10	Per-oral	Dieulafoy-like lesion on the fold	Hemoclipping	9	No recurrence

SRH: Stigmata of recent hemorrhage.

It has been reported that most patients with jejunal diverticular hemorrhage are treated with surgical intervention[7,15]. Jejunal diverticula are frequently seen in the sixth and seventh decades of life, as an acquired lesion with no muscularis layer on the mesenteric side. Patients undergoing surgery for duodenal diverticula are at risk for operative mortality and morbidity, as well as postoperative ileus and complications of general anesthesia[9].

Many modalities have been introduced to evaluate obscure gastrointestinal bleeding, including barium contrast study, CT scan, angiography, and CE. However, they have some limitations. The yield of CT scan for jejunal diverticula is only 2%[1]. Although the yield of CE for obscure gastrointestinal bleeding is 30%-80%[14], CE is not a therapeutic modality for obscure gastrointestinal bleeding and may result in retention of the capsule in diverticulum[16], as occurred in two of our patients. Barium contrast study has a high yield for detection of jejunal diverticula, but it is not a therapeutic modality for gastrointestinal bleeding. It has been reported that angiography plays a role in the diagnosis and treatment of small-bowel bleeding[17,18]. However, this procedure has limitations, including the risk of ischemic damage to the small-bowel wall, and failure to detect all bleeders.

It has been recently reported that DBE, a novel and unique technique, can successfully treat jejunal diverticular bleeding[19,20], and allow to-and-fro observation of the small intestine, and more importantly, permits tissue diagnosis and therapeutic intervention[11,21,22]. Thus, the aim of our study was to evaluate the efficacy of DBE therapy for jejunal diverticular bleeding.

Since endoscopic injection in treatment of nonvariceal hemorrhage was first described by Soehendra *et al*[23] in 1985, many endoscopic therapeutic modalities including contact or noncontact thermal therapy, and mechanical therapy by deploying a hemoclip on the feeding vessel have been developed for upper and lower gastrointestinal hemorrhage[24].

To our knowledge, this is the first case series report of patients with jejunal diverticular bleeding treated *via* endoscopy with diluted epinephrine local injection or hemoclips. The complication of injection therapy for small intestine lesions has been reported by Yen *et al*[25] who reported a case of intestinal wall necrosis after injection therapy. In addition, he reminds us that advanced age, atherosclerotic vascular disease, anemia, hypoxemia, shock and thinner intestinal wall are the risk factors. The amount of diluted epinephrine used in this study ranged 1-3 mL and no intestinal necrosis was found in patients. Nevertheless, further study is needed to determine the ideal amount of diluted epinephrine.

In this study, the mean follow-up time of patients was 14.7 ± 7.8 mo, during which only two patients had surgical intervention due to the large diverticulum and massive bleeding. One patient had recurrent ulcer bleeding after 7 mo, and was successfully treated with a repeat DBE by local injection therapy. No additional bleeding occurred in the other seven patients during the most recent follow-up.

In conclusion, DBE is a safe and effective treatment modality for jejunal diverticular bleeding. If rebleeding occurs, a repeat endoscopic therapeutic procedure should be attempted in addition to surgical intervention.

COMMENTS

Background

Jejunal diverticular massive bleeding is a rare complication of small bowel disease which usually takes the form of obscure gastrointestinal bleeding. Double-balloon enteroscopy (DBE) has been documented to be an effective tool for diagnosing and treating obscure gastrointestinal bleeding.

Research frontiers

The efficacy of endoscopy in diagnosing and treating jejunal diverticular bleeding was evaluated.

Innovations and breakthroughs

According to the findings in this study, DBE is a safe and effective treatment modality for jejunal diverticular bleeding.

Applications

DBE should be considered as the primary treatment modality for small intestine bleeding including jejunal diverticular bleeding.

Terminology

DBE uses 2 soft latex balloons fitted on tip of the enteroscope and overtube to complete the whole small bowel study.

Peer review

This is an interesting study describing the use of double-balloon-enteroscopy for hemorrhage from small bowel diverticula.

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BRIEF ARTICLE

Neoadjuvant chemotherapy for advanced gastric cancer: A meta-analysis

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Abstract

AIM: To study the value of neoadjuvant chemotherapy (NAC) for advanced gastric cancer by performing a meta-analysis of the published studies.

METHODS: All published controlled trials of NAC for advanced gastric cancer *vs* no therapy before surgery were searched. Studies that included patients with metastases at enrollment were excluded. Databases included Cochrane Library of Clinical Comparative Trials, MEDLINE, Embase, and American Society of Clinical Oncology meeting abstracts from 1978 to 2010. The censor date was up to April 2010. Primary outcome was the odds ratio (OR) for improving overall survival rate of patients with advanced gastric cancer. Secondary outcome was the OR for down-staging tumor and increasing R0 resection in patients with advanced gastric cancer. Safety analyses were also performed. All calculations and statistical tests were performed using RevMan 5.0 software.

RESULTS: A total of 2271 patients with advanced gastric cancer enrolled in 14 trials were divided into NAC group (n = 1054) and control group (n = 1217). The patients were followed up for a median time of 54 mo. NAC significantly improved the survival rate [OR = 1.27, 95% confidence interval (CI): 1.04-1.55], tumor stage (OR = 1.71, 95% CI: 1.26-2.33) and R0 resection rate (OR = 1.51, 95% CI: 1.19-1.91) of patients with advanced gastric cancer. No obvious safety concerns were raised in these trials.

CONCLUSION: NAC can improve tumor stage and survival rate of patients with advanced gastric cancer with a rather good safety.

Key words: Gastric cancer; Neoadjuvant chemotherapy; Survival; Meta-analysis

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INTRODUCTION

Gastric cancer is still a major health problem and a leading cause of cancer-related death although its incidence is decreased worldwide[1]. Surgery is the only curative treatment modality for gastric cancer and the overall survival (OS) rate of early-stage gastric cancer patients is up to 90%[2]. However, as the majority of gastric cancer patients are at the advanced stage at the time of diagnosis, their overall prognosis is suboptimal despite aggressive treatment, with an overall survival rate of 20%-30% after radical surgical

resection in Europe and about 60% in Japan[3,4]. Clearly, effective adjuvant therapy is needed to improve the outcome of patients with advanced gastric cancer.

Adjuvant therapy for gastric cancer has been extensively studied[2,5,6]. The effect of adjuvant chemoradiation therapy and perioperative chemotherapy has been demonstrated in well designed, multicenter and randomized clinical trials. It was recently reported that adjuvant chemotherapy has an affirmative effect on locally advanced gastric cancer[5]. S-1, an oral fluoropyrimidine (Taiho Pharmaceutical) used as an adjuvant chemotherapeutic agent, can increase 10% 3-year overall survival rate of patients with gastric cancer[6]. Among the potential strategies for adjuvant therapy, preoperative chemotherapy may provide an equal therapeutic efficacy as postoperative chemotherapy for gastric cancer[7]. Chemotherapy delivery may be more efficient if given prior to surgical disruption of vasculature, tumor down-staging may substantially facilitate surgical resection[8], and preoperative chemotherapy can be used to evaluate tumor chemosensitivity to cytotoxic medications. Furthermore, gastric cancer patients may tolerate preoperative cytotoxic treatment better than postoperative treatment, as performance status is usually negatively impacted by surgery[9]. However, lack of response to neoadjuvant chemotherapy (NAC) may delay curative surgery and chemotherapy-induced toxicity may increase surgical complications[10].

The effect of NAC on gastric cancer has been studied in several prospective trials[11-14]. However, no definite conclusion has been drawn from these trials. The underlying reasons included insufficient statistical power due to a limited sample size, an extended period of time for patient accrual, imbalanced treatment arms, and non-protocol treatment strategy. A well-designed randomized clinical trial is therefore needed to define the effect of NAC on advanced gastric cancer. A relatively effective alternative to provide clinical evidence under such circumstances is to perform a meta-analysis of the published clinical trials[15]. The current meta-analysis was to evaluate the role of NAC in treatment of gastric cancer and explore the optimal strategy for chemotherapy delivery.

MATERIALS AND METHODS

Inclusion criteria

All published controlled trials comparing NAC *vs* no treatment before surgery in patients with locally advanced gastric cancer were included in our analysis. Blindness of the trial was not necessary. The inclusion criteria were patients with pathologically diagnosed gastric adenocarcinoma and no history of prior treatment before entering the trial but a history of potentially curative surgery. A study was chosen if it was updated. NAC was performed through oral or intravenous (IV), intraperitoneal (IP) and intra-arterial (IA) infusion. Studies on preoperative radiotherapy or immunotherapy were excluded. Postoperative therapies were not included in our study.

Outcome

Primary outcome was the odds ratio (OR) of intervention with overall survival rate. Secondary outcome was the OR of R0 resection rates and tumor down-staging, which was represented as the percentage in stage pT0-2 after surgery. If the 95% confidence interval (CI) of the OR included 1.0, no difference was considered between the groups. Subgroup analysis was used to explore and explain the differences in results of different studies. Sensitivity analysis was also performed to show the publication bias.

Search method

Cochrane archives of clinical comparative trials, MEDLINE, Embase and American Society of Clinical Oncology meetings were retrieved from 1978 to 2010, with a censor date up to April 2010. The search strategy terms used in the English databases were "neoadjuvant chemotherapy OR preoperative chemotherapy" AND "gastric cancer OR stomach cancer OR stomach neoplasm OR gastric carcinoma" AND "clinical trial". Trials published in journals or published as meeting abstracts with essential data were included. Non-controlled trials were excluded. Randomized controlled trials with three or more arms were retained if at least two arms addressed an eligible comparison. Studies on patients with metastatic gastric cancer at enrollment were excluded.

Data checking

Methodological quality of trials was evaluated according to the Jadad quality scores[16], which include secure method of randomization, allocation concealment, patient and observer blinding, and losses to follow-up. Based on these criteria, the studies were divided into high quality group (score $\geqslant$ 4) and low quality group (score < 4). Two reviewers independently assessed the eligibility of each trial.

Data extraction

Authors, year of publication, country of investigators, sample size (total, eligible, and per arm), chemotherapy regimen, cycles of chemotherapy, follow-up period, curative effect (survival rate, rate of macroscopic radical resection cancer stage at pathological examination), and adverse events of each eligible trial were recorded. Two reviewers independently made extracts from each study.

Statistical analysis

RevMan software 5.0 was employed for the meta-analysis[17]. Continuous data were expressed as weighted mean difference. OR for dichotomous parameters including overall survival rate, tumor down-staging, and R0 resection rate was recorded. Results were reported as 95% CI. All meta-analyses appraised inter-study heterogeneity using χ^2-based Q statistics for statistical significance and I^2 statistics for the degree of heterogeneity. $P < 0.10$ was considered statistically significant and $I^2 > 50\%$ showed a large heterogeneity. If there was no heterogeneity, a fixed-effect model was used. Otherwise, a random-effect model was used. The number needed to treat (NNT) was applied for outcomes with a statistical difference. Publishing bias was tested using the funnel plot. Sensitivity analysis was performed to investigate the possible influence of the study quality on the results. The main outcome of high-

Table 1 Basic characteristics of trials included in this study

Author and year of publication (citation)	Country	Patients (*n*)		NAC group		Control group		Median follow-up (mo)
		NAC	Control	Pre-op	Post-op	Pre-op	Post-op	
Schuhmacher *et al*[18], 2009	Germany	72	72	5-FU + DDP	None	None	None	53
Boige *et al*[19], 2007	France	113	111	FP	FP	None	None	68
Cunningham *et al*[20], 2006	UK	250	253	ECF	ECF	None	None	47
Hartgrink *et al*[21], 2004	Holland	27	29	FAMTX	None	None	None	83
Nio *et al*[22], 2004	Japan	102	193	UFT (oral)	CT	None	CT	83
Zhang *et al*[23], 2004	China	37	54	IV (no details)	None	None	None	NR
Kobayashi *et al*[24], 2000	Japan	91	80	5-FU (oral)	CT	None	None	NR
Wang *et al*[25], 2000	China	30	30	5-FU (oral)	None	None	None	NR
Takiguchi *et al*[26], 2000	Japan	123	139	5-FU ± DDP	None	None	None	NR
Lygidakis *et al*[27], 1999	Greece	39	19	IP (no details)	CT	None	None	NR
Kang *et al*[28], 1996	Korea	53	54	PEF	PEF	None	PEF	> 36
Masuyama *et al*[29], 1994	Japan	24	98	EAP (IA)	None	None	None	> 36
Yonemura *et al*[30], 1993	Japan	29	26	PMUE	None	None	PMUE	24
Nishioka *et al*[31], 1982	Japan	64	59	5-FU (oral)	CT	None	CT	> 60

NAC: Neoadjuvant chemotherapy; 5-FU: 5-fluorouracil; DDP: Cisplatin; FP: 5-FU/cisplatin; ECF: Epirubicin/cyclophosphamide/5-FU; FAMTX: 5-FU/adriamycin/methotrexate; UFT: Tegafur/uracil; CT: Chemotherapy; IV: Intravenous; IP: Intraperitoneal; PEF: Cisplatin/epirubicin/5-FU; EAP: Epirubicin/adriamycin/cisplatin; PMUE: Cisplatin/mitomycin C/etoposide/UFT.

Table 2 Quality assessment of trials included in this study

Author and year of publication (citation)	Randomization	Allocation concealment	Blind	Withdrawal and dropout	Jadad score
Schuhmacher *et al*[18], 2009	Without details	Without details	Without details	Well reported	4
Boige *et al*[19], 2007	Without details	Without details	Without details	Well reported	4
Cunningham *et al*[20], 2006	Well reported	Envelope	Double-blind	Well reported	7
Hartgrink *et al*[21], 2004	Well reported	Envelope	No	Well reported	5
Nio *et al*[22], 2004	Inappropriate	None	No	Well reported	1
Zhang *et al*[23], 2004	Without details	None	No	Well reported	2
Kobayashi *et al*[24], 2000	Well reported	Envelope	No	Well reported	5
Wang *et al*[25], 2000	Without details	Without details	No	Well reported	3
Takiguchi *et al*[26], 2000	Without details	None	No	Well reported	2
Lygidakis *et al*[27], 1999	Inappropriate	Without details	No	Well reported	2
Kang *et al*[28], 1996	Without details	Without details	No	Well reported	3
Masuyama *et al*[29], 1994	Inappropriate	None	No	Well reported	1
Yonemura *et al*[30], 1993	Well reported	Envelope	No	Well reported	5
Nishioka *et al*[31], 1982	Inappropriate	None	No	Well reported	1

quality trials was analyzed. Subgroup analysis was used to explore and explain the diversity in results of different studies for collecting information with different characteristics, such as tumor grade, chemotherapy regimen, and race.

RESULTS

Eligible trials

A total of 354 studies were retrieved. After abstracts were read, 335 studies were found to be unrelated to our selection criteria and it was impossible to extract data from another 5 studies. Thus, only 14 studies[18-31] were eligible for our meta-analysis. The enrolled 2271 gastric cancer patients in the studies were divided into NAC group (n = 1054) and control group (n = 1217). Of the 14 studies, 9 were from Asian and 5 from Western countries. NAC routes were IV, IA, IP, and oral in 7, 2, 1, and 4 studies, respectively. The median follow-up time of the patients across the studies was 54 mo (Table 1). The quality of included studies was assessed according to the Jadad quality scores[16] for the 4 requirements (method of randomization, allocation concealment, blindness, and completeness of follow up). Accordingly, 6 studies had a score greater than 4 (high quality, low risk of bias)[18-21,24,30] (Table 2).

Overall survival rates

Twelve studies[18-25,27,29-31] with 1868 patients (878 in NAC group and 990 in control group) reported survival rates at the end of follow-up. The median follow-up time in these studies was over 3 years. The NAC group had a marginal survival benefit compared to the control group (48.1% *vs* 46.9%, respectively), with an OR of 1.27 (95% CI: 1.04-1.55, fixed-effect model) and a NNT of 84 (Figure 1A).

Three-year progression-free survival rate

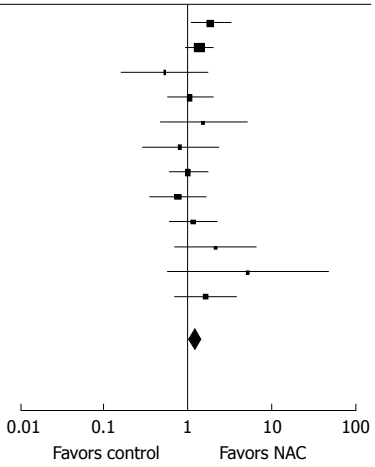
Three studies[18-20] compared the 3-year progression-free survival (PFS) rates for the two groups. The 3-year PFS

A Overall survival rate

Study	NAC Events	NAC Total	Control Events	Control Total	Odds ratio M-H, fixed (95% CI)
Boige *et al*[19], 2007	43	113	27	111	
Cunningham *et al*[20], 2006	101	250	83	253	
Hartgrink *et al*[21], 2004	6	27	10	29	
Kobayashi *et al*[24], 2000	57	91	49	80	
Lygidakis *et al*[27], 1999	14	39	5	19	
Masuyama *et al*[29], 1994	18	24	58	74	
Nio *et al*[22], 2004	73	102	137	193	
Nishioka *et al*[31], 1982	34	64	29	49	
Schuhmacher *et al*[18], 2009	40	72	37	72	
Wang *et al*[25], 2000	12	30	7	30	
Yonemura *et al*[30], 1993	5	29	1	26	
Zhang *et al*[23], 2004	19	37	21	54	
Total (95% CI)		878		990	
Total events	422		464		

Heterogeneity: $\chi^2 = 10.28$, $df = 11$ ($P = 0.51$); $I^2 = 0\%$

Test for overall effect: $Z = 2.39$ ($P = 0.02$)

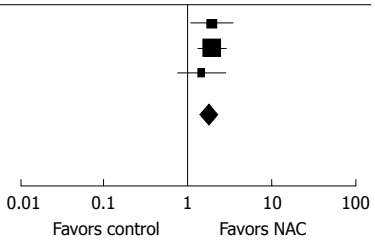


B 3-yr PFS

Study	NAC Events	NAC Total	Control Events	Control Total	Odds ratio M-H, fixed (95% CI)
Boige *et al*[19], 2007	45	113	28	111	
Cunningham *et al*[20], 2006	98	250	63	253	
Schuhmacher *et al*[18], 2009	36	72	29	72	
Total (95% CI)		435		436	
Total events	179		120		

Heterogeneity: $\chi^2 = 0.54$, $df = 2$ ($P = 0.76$); $I^2 = 0\%$

Test for overall effect: $Z = 4.23$ ($P < 0.0001$)

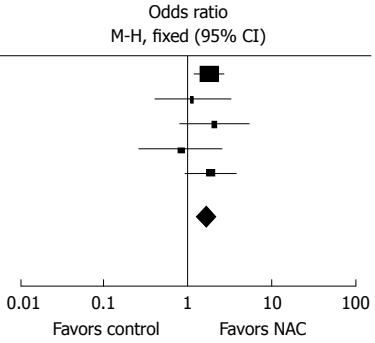


C Tumor down-staging

Study	NAC Events	NAC Total	Control Events	Control Total	Odds ratio M-H, fixed (95% CI)
Cunningham *et al*[20], 2006	89	172	71	193	
Hartgrink *et al*[21], 2004	15	27	15	29	
Kang *et al*[28], 1996	14	47	9	54	
Lygidakis *et al*[27], 1999	13	39	7	19	
Schuhmacher *et al*[18], 2009	46	70	34	68	
Total (95% CI)		355		363	
Total events	177		136		

Heterogeneity: $\chi^2 = 2.33$, $df = 4$ ($P = 0.68$); $I^2 = 0\%$

Test for overall effect: $Z = 3.45$ ($P = 0.0006$)

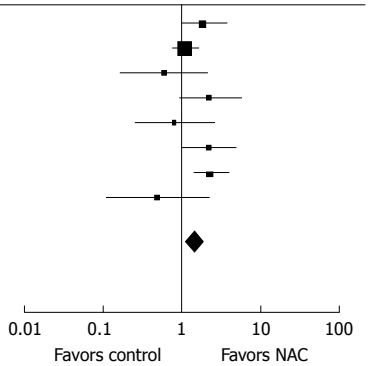


D Resection rate

Study	NAC Events	NAC Total	Control Events	Control Total	Odds ratio M-H, fixed (95% CI)
Boige *et al*[19], 2007	95	113	81	111	
Cunningham *et al*[20], 2006	169	244	166	250	
Hartgrink *et al*[21], 2004	20	27	24	29	
Kang *et al*[28], 1996	37	47	33	54	
Lygidakis *et al*[27], 1999	25	39	13	19	
Schuhmacher *et al*[18], 2009	59	72	48	72	
Takiguchi *et al*[26], 2000	94	123	80	139	
Yonemura *et al*[30], 1993	23	29	23	26	
Total (95% CI)		694		700	
Total events	522		468		

Heterogeneity: $\chi^2 = 12.68$, $df = 7$ ($P = 0.08$); $I^2 = 45\%$

Test for overall effect: $Z = 3.43$ ($P = 0.0006$)



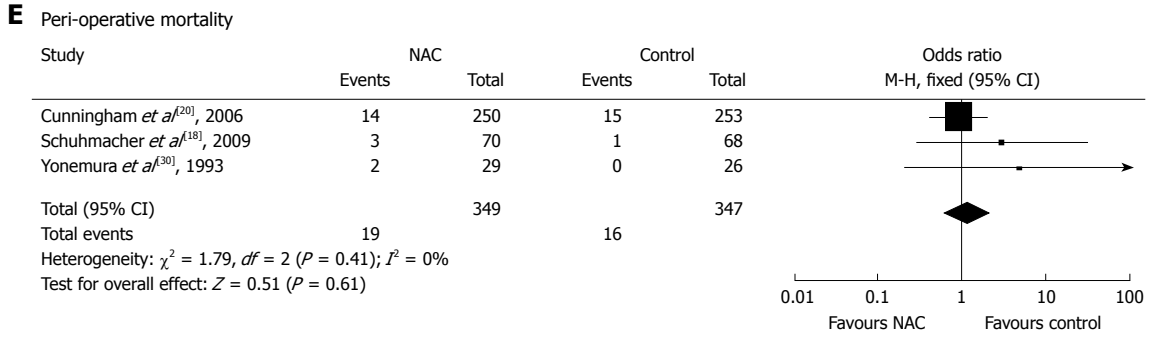


Figure 1 Overall survival rates of patients with advanced gastric cancer in 12 studies (A), 3-year progression-free rates in 3 studies (B), tomor down-staging rates in 6 studies (C), R0 resection rates in 8 studies (D), and adverse effects of neoadjuvant chemotherapy (E). NAC: Neoadjuvant chemotherapy; PFS: Progression-free survival; CI: Confidence interval.

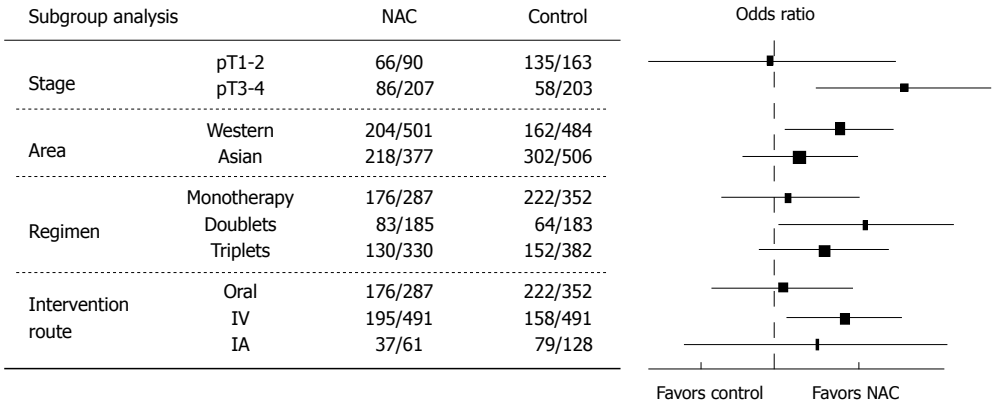


Figure 2 Subgroup analysis showing different overall survival rates of patients with advanced gastric cancer in trials from Western and Asian counties. NAC: Neoadjuvant chemotherapy; IV: Intravenous; IA: Intra-arterial.

rate was higher for NAC group than for control group (41.1% *vs* 27.5%), with an OR of 1.85 (95% CI: 1.39-2.46, fixed-effect model) and a NNT of 8 (Figure 1B).

Tumor down-staging rate

Six studies[18,20-22,27,28] described the pathological staging of gastric cancer after resection. Except for one study[22] showing non-comparative staging data at baseline from the two groups, the other 5 studies involving 718 patients (355 in NAC group and 363 in control group) were included in the final analysis. The rate of pT0-2 was higher for NAC group than for control group (49.9% *vs* 37.5%), suggesting that NAC has a significant down-staging effect on gastric cancer with an OR of 1.71 (95% CI: 1.26-2.33) and a NNT of 9 (Figure 1C).

R0 resection rate

The resection rate of gastric cancer was reported in 8 trials[18-21,26-28,30]. Since no obvious heterogeneity was observed in these studies (P = 0.08, I^2 = 45%), the fixed-effect model was used. The R0 resection rate of gastric cancer was higher for NAC group than for control group (75.2% *vs* 66.9%) with an OR of 1.51 (95% CI: 1.19-1.91, fixed-effect model) (Figure 1D).

Safety analysis

Safety analysis included both chemotherapy-induced adverse effects (grade 3/4, defined according to the Common Toxicity Criteria of the National Cancer Institute, version 2.0) and perioperative morbidity and mortality. Three studies[20,22,30] reported grade 3/4 adverse effects of NAC, including gastrointestinal (GI) problems in 8.8% (31/353) and leukopenia in 18.1% (62/343) of gastric cancer patients. Three studies[18,20,30] reported perioperative mortality with no statistically significant difference (P = 0.61) between the two groups (5.4% *vs* 4.6%, Figure 1E).

Subgroup analysis

Different factors that might be related to the different results between the two groups were studied (Figure 2). When the overall survival rate was set as the end point, gastric cancer patients at a later stage (pT3-4) benefited more from NAC than those at an earlier stage (pT1-2) (OR = 1.91, 95% CI: 1.24-2.96, NNT = 8). Trials from Western countries showed more solid data favoring NAC than those from Asian countries (OR = 1.39, 95% CI: 1.07-1.80). Monotherapy regimens were inferior to doublet or triplet chemotherapy regimens (OR = 1.05, 95% CI: 0.75-1.48). IV route of NAC was better than other routes (OR = 1.42, 95% CI: 1.08-1.85).

DISCUSSION

Our current meta-analysis demonstrated the feasibility

of NAC for locally advanced gastric cancer. NAC could down-stage (NNT = 9) and increase the R0 rate of gastric cancer. However, whether NAC improves the overall survival rate of gastric cancer patients is still controversial[5]. To examine the role of NAC alone in improving the overall survival rate of gastric cancer patients who did not receive postadjuvant chemotherapy, data from the 5 trials[18,21,23,25,29] were further analyzed, showing that NAC has no effect on the overall survival rate of gastric cancer patients (OR = 1.20, 95% CI: 0.8-1.80). Since all the included studies had a very small sample size (17-123 patients in the NAC arms) with a different follow-up time, no clear conclusion could be reached on the effect of NAC alone on overall survival rate of gastric cancer patients.

Our meta-analysis showed that gastric cancer patients could well tolerate NAC. Preoperative chemotherapy was feasible as over 80% patients with advanced gastric cancer completed all treatment courses, except for one trial where only 55.6% of the patients completed all courses[21]. Grade 3/4 GI adverse events of NAC occurred in 8.8% (31/353) of gastric cancer patients, which is considerably lower than that (25%) induced by postoperative adjuvant chemotherapy[32]. The tolerance to postoperative adjuvant chemotherapy was often marred by surgery-related GI effects. No severe and obvious postoperative complications, such as anastomotic leakage, infection, or death, occurred after NAC, indicating that NAC is a safe modality for gastric cancer.

Overtreatment of early gastric cancer with NAC is a potential concern. Whether later stage gastric cancer (T3-4) is the optimal group for NAC was analyzed. A Japanese trial[2] on serosa-negative cancer including node-positive disease showed that the 5-year disease-free survival rate and overall survival rate are 83% and 86%, respectively for gastric cancer patients receiving D2 surgery (gastrectomy with extended lymph-node dissection), indicating that early-stage gastric cancer patients may be cured with adequate surgical therapy alone rather than with NAC. Subgroup analysis also showed that the outcome of NAC for gastric cancer was better in trials from Western countries than in those from Asian countries. Of the 14 trials included in the present study, 6 were from Japan that included more patients with gastric cancer diagnosed at an earlier stage, thus possibly influencing the final results.

One of the questions concerning NAC for gastric cancer is its best regimen. Great efforts have been made on finding the optimal NAC for gastric cancer. In our current meta-analysis, few data were available to answer this question. However, our analysis showed that combination regimen and IV route of NAC had a high efficiency on advanced gastric cancer. Theoretically, NAC with a high response rate can be recommended for metastatic gastric cancer. For example, docetaxel-based[33] or epirubicin-based triple therapy[34] is a good option for metastatic gastric cancer. Regimens with a rapid and high response rate help to down-stage tumors to the greatest extent and increase the probability of R0 resection, thus improving the survival rate for patients with gastric cancer.

Disease progression during NAC is another potential concern due to a loss of opportunity for surgery. In our meta-analysis, two trials[18,22] showed a disease progression rate of 2.9% (4/138). Of the 402 patients with gastric cancer, 108 (26.9%) in NAC group underwent palliative resection (n = 76) or with their tumor unresectable (n = 32), which was lower than that (33.8%) in control group[18,20-22,30], indicating that disease progression after NAC is not a major concern for its resection.

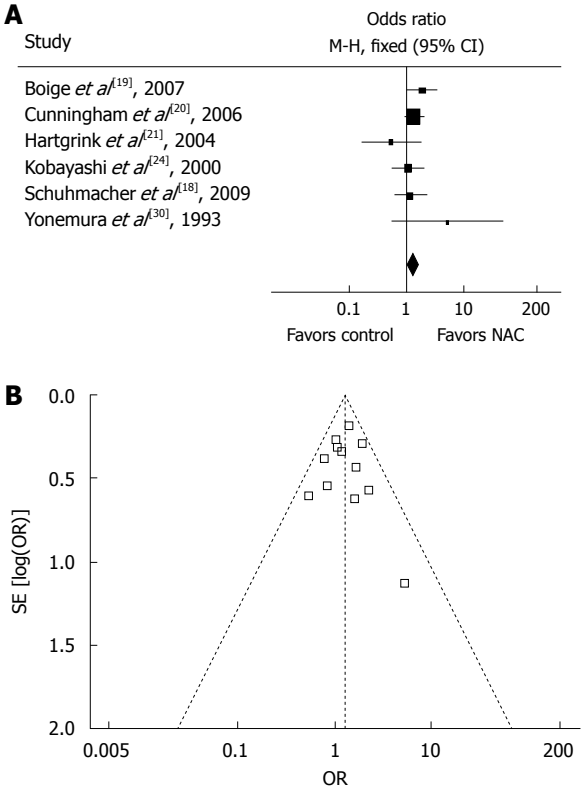


Figure 3 Odds ratio (A) and publication bias (B) in studies included in this study. A: Sensitivity analysis for overall survival: high-quality studies (Jadad score ⩾ 4); B: Funnel plot analysis of potential publication bias.

A major concern in our meta-analysis was the quality of studies included. Since no sufficient randomized controlled trials were available on NAC for gastric cancer, our current meta-analysis included quasi-randomized or non-randomized but controlled trials with basic data that could be used for comparison. Sensitivity analysis was therefore performed to determine the effect of trial quality on the final results. Six high-quality trials with a Jadad score ⩾ 4[18-21,24,30] reported the overall survival rate with an OR of 1.35 (95% CI: 1.06-1.73) compared to that with an OR of 1.27 (95% CI: 1.04-1.55) in all the studies (Figure 3A), indicating that the results are consistent with those in all the trials and are therefore independent of trial quality. Furthermore, the symmetrical shape of 'funnel plot' when drawing together survival rate and sample size for all the studies indicated that no obvious publication bias was found in our meta-analysis (Figure 3B).

NAC has been proven effective against some cancers, such as breast cancer[35]. However, it is not generally recommended for gastric cancer, primarily because of differences in treatment modalities for gastric cancer

between Asian and Western countries. A Western study demonstrated that D2 surgery can effectively remove lymph nodes[36], whereas a Japanese study showed that D2 surgery is highly effective and safe, and recommended as a routine surgery[4]. It was reported that chemoradiotherapy is the standard therapy for gastric cancer after operation in USA[37] and most appropriate for the D0 dissection population. Whether post-chemoradiotherapy benefits D2 dissection patients lacks strong evidence. It has been shown that perioperative chemotherapy can prolong the survival time of locally advanced gastric cancer patients who tolerate preoperative chemotherapy better than postoperative chemotherapy[20]. Therefore, NAC is a hopefully good option for locally advanced gastric cancer, although further studies are required to determine its best regimen.

Our meta-analysis provided the up-to-date evidence for the positive effect of NAC on locally advanced gastric cancer. A number of new trials have been registered to examine the role of NAC in treatment of advanced gastric cancer, such as S-1 plus cisplatin[38]. Our meta-analysis should therefore be further updated whenever new and strong evidence is available. With the increasing acceptance of the concept of NAC, additional studies on new regimens and well-designed powerful trials are highly encouraged in patients with locally advanced gastric cancer.

ACKNOWLEDGMENTS

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COMMENTS

Background

Over 50% of patients with newly diagnosed gastric cancer have advanced disease. Even after surgery, their prognosis remains poor. The effect of chemotherapy on advanced gastric cancer has been proven. Neoadjuvant chemotherapy (NAC) plays a role in improving the prognosis of advanced gastric cancer patients, but its value remains controversial because of lack of well-powered trials.

Research frontiers

Meta-analysis was used to evaluate the value of NAC for advanced gastric cancer in this study.

Innovations and breakthroughs

The meta-analysis provided the up-to-date evidence for the positive effect of NAC on locally advanced gastric cancer. NAC improved the R0 resection rate (95% CI: 1.19-1.91), tumor down-staging (95% CI: 1.26-2.33) and survival rate (95% CI: 1.04-1.55) for the 2271 patients enrolled in 14 trials. No obvious safety concerns were raised in these trials. These findings suggest that NAC can improve the survival rate of patients with advanced gastric cancer.

Applications

With the increasing acceptance of the concept of NAC, additional studies on new regimens and well-designed powerful trials are highly encouraged in patients with locally advanced gastric cancer.

Terminology

MAGIC trial: A phase Ⅲ clinical trial conducted by the Medical Research Council Adjuvant Gastric Infusional Chemotherapy, showing the effect of perioperative chemotherapy on advanced gastric cancer. INT-0116 study: The Intergroup-0116 study reporting that postoperative chemoradiotherapy is effective against gastric adenocarcinoma or gastroesophageal junction. D2 surgery: D2 lymphadenectomy which was defined according to the rules of the Japanese Research Society for Gastric Cancer and, therefore, included all lymphnodes of levels N1 and N2. Adverse events: Assessed according to the Common Toxicity Criteria of the National Cancer Institute (version 2.0) and defined as grade 0-4. Tumor stage: Assessed according to the International Union against Cancer: TNM classification of malignant tumors. "pT" indicates the pathological stage after surgery and "cTNM" indicates the clinical pretreatment tumor stage. S-1: An orally active combination of tegafur (a prodrug that is converted by cells to fluorouracil), gimeracil (an inhibitor of dihydropyrimidine dehydrogenase, which degrades fluorouracil), and oteracil (which inhibits the phosphorylation of fluorouracil in the gastrointestinal tract, thereby reducing the gastrointestinal toxic effects of fluorouracil) with a molar ratio of 1:0.4:1.

Peer review

This is an important analysis of NAC for advanced gastric cancer. The meta-analysis of all available controlled trials on NAC conduced by the authors may provide the up to date evidence of NAC for advanced gastric cancer.

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BRIEF ARTICLE

Screening test for anti-*Helicobacter pylori* activity of traditional Chinese herbal medicines

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Abstract

AIM: To evaluate the anti-*Helicobacter pylori* (*H. pylori*) activity of 50 traditional Chinese herbal medicines in order to provide the primary evidence for their use in clinical practice.

METHODS: A susceptibility test of water extract from 50 selected traditional Chinese herbal medicines for *in vitro H. pylori Sydney strain 1* was performed with broth dilution method. Anti-*H. pylori* activity of the selected Chinese herbal medicines was evaluated according to their minimum inhibitory concentration (MIC).

RESULTS: The water extract from Rhizoma Coptidis, Radix Scutellariae and Radix isatidis could significantly inhibit the *H. pylori* activity with their MIC less than 7.8 mg/mL, suggesting that traditional Chinese herbal medicines have anti-inflammatory and antibacterial effects and can thus be used in treatment of *H. pylori* infection.

CONCLUSION: Rhizoma Coptidis, Radix Scutellariae and Radix isatidis are the potential sources for the synthesis of new drugs against *H. pylori*.

Key words: Chinese herbal medicines; *Helicobacter pylori*; Minimum inhibitory concentration; Gastric; Oral

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INTRODUCTION

Helicobacter pylori (*H. pylori*), a microaerophilic, Gram-negative spiral bacterium which was first detected in 1984 by Marshall *et al*[1], is one of the most common chronic bacterial pathogens in humans. Approximately 50% of people in the world are infected with it, and its prevalence is significantly higher in developing countries than in developed countries[2]. *H. pylori* infection is an important etiologic impetus usually leading to chronic gastritis, gastroduodenal ulcer and low grade gastric mucosa-associated lymphoid tissue lymphoma. Epidemiological data show that a high *H. pylori* infection rate is related to the high incidence of gastric cancer and gastric adenocarcinoma[3]. World Health Organization has categorized *H. pylori* as

a class 1 carcinogen[4]. Fortunately, its eradication with antibiotics can result in ulcer healing, prevent peptic ulcer recurrence and reduce the prevalence of gastric cancer in high-risk populations[5]. However, it is not always successful because of its resistance to one or more antibiotics and other factors such as poor patient compliance, undesirable side effects of the drugs and significant cost of combination therapy. Worrel *et al*[6] reported that over 15% of the patients undergoing antibiotics therapy would experience therapeutic failure. In developing countries, since the application of antibiotics is still under a poor management as a whole, there is a growing need for finding new anti-*H. pylori* agents that can hopefully eradicate the invasion and presence of survived *H. pylori* strains to avoid relapse of gastric ulcer. Hence, a considerable variety of studies involving tests for medicinal plants showing antimicrobial activity and discrepant susceptibility test results are available due to variations in the methods and conditions used for its susceptibility testing. It was reported that Garlic extracts exhibit a weak or modest anti-*H. pylori* activity[7,8]. Pteleopsis suberosa[9], Cinnamon[10], Cranberry juice[11], Aristolochia paucinervis Pomel[12], black Myrobalan[13], *etc.,* have also been found to have anti-*H. pylori* activities. Ndip *et al*[14] reported that Ageratum conyzoides, Scleria striatinux and Lycopodium cernua show a very potent antibacterial activity. Fifty-four herbal medicines from Korea have been screened for their anti-*H. pylori* activity, of which, Rheum palmatum, Rhus javanica, Coptis japonica and Eugenia caryophyllata have a strong anti-*H. pylori* activity[15]. Extracts and fractions from 7 Turkish plants also demonstrate anti-*HP* activities[16]. Traditional medicinal plants from Pakistan and Psoralea corylifolia L. demonstrate a strong anti-*H. pylori* activity[17]. Some compounds even have been isolated and their anti-*H. pylori* activity has also been testified, for example, Myroxylon Peruiferum from the Brazilian medicinal plants[18]. In addition, some flavonoids and isoflavonoids isolated from licorice, such as licochalcone A and licoisoflavone B, have been reported to exhibit inhibitory activities against *H. pylori*[19].

In China, traditional Chinese medicine and pharmacology play an indispensable role in the health care system, especially in prevention and management of chronic diseases. Studies[20,21] revealed that some traditional herbal medicines are efficient against gastrointestinal diseases, including chronic gastritis and peptic ulcer disease, a major outcome of *H. pylori* infection, indicating that the medicinal plants may contain constituents, which have antibacterial and anti-inflammatory activities. The present study was to evaluate the anti-*H. pylori* activity of some selected medicinal plants to identify the potential sources for synthesis of new drugs against *H. pylori*. In this study, 50 traditional Chinese medicinal herbs (Table 1) were examined and screened for their anti-*HP* activity according to their minimum inhibitory concentration (MIC).

MATERIALS AND METHODS

Extract of medicinal plants

A total of 50 traditional Chinese medicinal herbs, purchased from Tongren Drugstore, were identified by Professor Chang-Qing Wang, Beijing Academy of Agriculture and Forestry Sciences. Medicinal herbs were selected according to their traditional use in Chinese pharmacology as anti-inflammatory or antibacterial drugs. Radix Scutellariae Baicalensis (20 g) was soaked for 2 h in about 1000 mL distilled water at room temperature, decocted for 1 h (first quickly, then slowly), and filtered through filter paper. Their residue was decocted for a second time and filtered. The filtrate was collected and centrifuged at 3500 r/min for 15 min. Finally, the liquid was concentrated to 10 mL (2 g/mL) in a rotary vacuum evaporator, then capped and autoclaved for 15 min at 121℃. Under the sterile environment, 12 sterile 7.5 cm × 1.3 cm capped tubes were arranged in a row in the rack, marked with No. 1, No. 2, …, No. 12 tubes, then 1 mL sterile distilled water was transferred to each tube with a fresh transferpettor and decocted. A Radix Scutellariae Baicalensis stock solution (2 g/mL) was added into No. 1 tube and mixed thoroughly. Then 1 mL solution was transferred from No. 1 tube to No. 2 tube, diluted twice until no extract dilution solution was added into the No. 10, No. 11 and No. 12 tubes as controls. After that, 1 mL two-fold concentration of modified brucella broth (control of diarrheal diseases in China Research, Shanghai Regent Supply) supplemented with 10% sterile fetal calf serum (Guangzhou Ruite Ltd., China) was added into each of the tubes. The final concentration of medicinal plants reached 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 2.0 and 1.0 mg/mL in No. 1-10 tubes, respectively. Extraction was performed in duplicate from other medicinal plants and their extracts were stocked at 4℃ prior to use.

Inocula

H. pylori Sydney strain 1 (*SS1*), obtained from Laboratory of Medical Microbiology, Southern Medical University, was stored in brain-heart infusion broth supplemented with 20% (v/v) glycerol and 1% yeast extract at -70℃. After freeze thawing under a superclean bench (Shanghai Anting Scientific Instrument Ltd., China), a drop of *H. pylori* was inoculated into a single-fold concentration of modified sterile brucella broth (as above), cultured at 37℃ in a shaking incubator containing 5% O_2, 10% CO_2, and 85% N_2 at 150 r/min for 3 d. *H. pylori SS1* was identified with routine diagnostic procedures, according to their colony morphology, Gram-staining, test of oxidase, catalase and urease reaction, as well as molecular identification based on the amplified species-specific sequences of 16s rRNA by polymerase chain reaction (PCR). Bacterial suspension was transferred quickly into 0.9% sterile physiological saline and diluted to the McFarland standard 0.5 (1×10^8 cfu/mL) as the standardized bacterial suspension. The turbidity was verified by spectrophotometrically measuring the absorbance of the suspension ($A_{625\ nm}$).

Susceptibility testing

Ten microliters of standardized bacterial suspension (1×10^8 cfu/mL) was inoculated into No. 1-11 tubes within

Table 1 Fifty anti-*Helicobacter pylori* traditional Chinese herbal medicines

Chinese name	English name	Pharmaceutical name	Botanical name	MIC (mg/mL)
Huanglian	Coptis Rhizome	Rhizoma Coptidis	Coptis chinensis Franch.	3.9 < MIC ≤ 7.8
Huangqin	Baikal skullcap Root	Radix Scutellariae	Scutellaria Baicalensis Georgi.	3.9 < MIC ≤ 7.8
Banlangen	Indigowoad Root	Radix isatidis	Isatis tinctoria L.	3.9 < MIC ≤ 7.8
Jinyinhua	Honeysuckle Flower	Floslonicerae Japonicae	L. Similis Hemsl	7.8 < MIC ≤ 15.6
Qinpi	Largeleaf Chinese Ash Bark	Cortex Fraxini	F. Bungeana DC.	7.8 < MIC ≤ 15.6
Zihuadiding	Tokyo Violet Herb	Herba Violae cum Radice	Viola yedoensis Mak.	15.6 < MIC ≤ 31.2
Huangbai	Chinese Corktree Bark	Cortex Phellodendri	Chinese Schneid	15.6 < MIC ≤ 31.2
Daqingye	Indigowoad Leaf	Foliun isatidis	Isatis	15.6 < MIC ≤ 31.2
Pugongying	Dandelion	Herba Taraxaci Mongolici cum Radice	Taraxacacum mongolicum	15.6 < MIC ≤ 31.2
Dahuang	Rhubarb	Rhizoma Rhei	R. Officinale baill	31.2 < MIC ≤ 62.5
Shandougen	Tonkin sophora Root	Radix Sophorae Tonkinensis	Sophora Subprostrata Chun et T. Chen	31.2 < MIC ≤ 62.5
Longdancao	Chinese Gentian Root	Radix Gentianae Scabrae	Gentiana Scabra Bge. In	31.2 < MIC ≤ 62.5
Hezi	Medicine Terminalia Fruit	Fructus Terminaliae Chebulae	Terminalia chebula Retz.	31.2 < MIC ≤ 62.5
Machixian	Purslane Herb	Radix Sophorae Subprostratae	Sophora Subprostrata Chun et T. Chen	62.5 < MIC ≤ 125
Banzhilian	Barbed Skullcap Herb	Herba Scutellariae Barbatae	Scutellaria Barbata D. Don	62.5 < MIC ≤ 125
Yuxingcao	Heartleaf Houttuynia Herb	Herba Houttuyniae Cordatae	Houttuynia cordata Thunb.	62.5 < MIC ≤ 125
Tufuling	Glabrous Greenbrier Rhizome	Rhizoma Smilacis Glabrae	Simlax glabra Roxb.	62.5 < MIC ≤ 125
Niubangzi	Great Burdock Achene	Fructus Arctii Lappae	Fructus Arctii	125 < MIC ≤ 250
Juhua	Chrysanthemum Flower	Flos Chrysanthemi Morifolii	Chrysanthemum morifolium Ramat.	125 < MIC ≤ 250
Baijiangcao	Whiteflower Patrinia Herb	Herba Whiteflower Patrinia Herb	Patrinia Scabiosaefolia Fisch.	125 < MIC ≤ 250
Tianhuafen	Snakegourd Root	Radix Trichosanthis	Thichosanthes kirilowii Maxim.	125 < MIC ≤ 250
Yadanzi	Java Brucea Fruit	Fructus Brucae Javanicae	Brucea Javanica Merr.	125 < MIC ≤ 250
Niuhuang	Cow-bezoar	Calculus Bovis	Bos taurus domesticus Gmelin	250 < MIC ≤ 500
Mabo	Puff-ball	Frucifictio lasiosphaerae	lasiosphara fenslli Reich.	250 < MIC ≤ 500
Zisu	Perilla Leaf	Folium Perillae Frutescentis	Perilla	250 < MIC ≤ 500
Chaihu	Chinese Thorowax Root	Radix Bupleuri	Bupleurum scorzoneraefolium	250 < MIC ≤ 500
Rendongteng	Honeysuckle Stem	Caulis Lonicerae	Lonicera Japonica Thunb.	250 < MIC ≤ 500
Kushen	Lightyellow Sophora Root	Radix Sophorae Flavescentis	Sophora Flavscens Ait	250 < MIC ≤ 500
Rougui	Cassia Bark	Cortex Cinamomi Cassiae	Cinnamomum cassia Presl.	> 500
Congbai	Fistular Onion Stalk	Herba Alii Fistulosi	Allium fistulosum L.	> 500
Xiangru	Haichow Elsholtzia Herb	Herba Elsholtziae Splendentis	E. Haichowensis Sun.	> 500
Bohe	Wild Mint	Herba Menthae	Mentha haplocalyx Briq.	> 500
Qinghao	Sweet Wormwood	Herba Artemisiae Apiaceae	Artemeisia apiacea Hance	> 500
Wuzhuyu	Medicinal Evodia Fruit	Fructus Evodiae Rutaecarpae	Evodia Rutaecarpa Benth.	> 500
Chishaoyao	Red Peony Root	Radix Paeoniae Rubra	Paeonia lactiflora Pall.	> 500
Wumei	Smoked Plum	Fructus Pruni Mume	Prunus mume Sieb. et Zucc	> 500
Mudanpi	Tree Peony Bark	Cortex Moutan Radicis	Paeonia suffruticosa Andr	> 500
Xuanshen	Figwort Root	Radix Scrophulariae Ningpoensis	Scrophularia ningpoensis Hemsl	> 500
Ganjiang	Dried Ginger	Rhizoma Zingiberis Officinalis	Zingiber officinalum Rose	> 500
Fuzi	Root of Common Monkshood	Rzdix Aconiti Carmichaeli Praeparata	Aconite carmichaeli Debx.	> 500
Huajiao	Bunge pricklyash	Fructus Zanthoxyli Bungeani	Zanthoxylum bungeanum Maxim.	> 500
Gaoliangjiang	Lesser Galangal Rhizome	Rhizoma Alpiniae Officinarum	Alpinnia offcinarum Hance.	> 500
Dingxiang	Clove Flower-bud	Flos Caryophylli	Eugenia caryophllata Thunb.	> 500
Shiliupi	Rind Peel	Pericarpium Punicae Granati	Punica granatum L.	> 500
Xixin	Manchurian wildginger Herb	Herba Asari cum Radice	Asarum sieboldii Miq.	> 500
Cangzhu	Swordlike Atractylodes Rhizome	Rhizoma Atractylodis	Atractylodes lanccea Thumb.	> 500
Lugen	Reed Rhizome	Rhizome Phragmitis Communis	Phragmites communis	> 500
Baitouweng	Chinese pulsatilla Root	Radix pulsatillae Chinensis	Pulsatilla Chinensis Reg.	> 500
Xiaohuixiang	Fennel Fruit	Fructus Foeniculi vulgaris	Foeniculum vulgare Mill.	> 500
Zhizi	Cape Jasmine Fruit	Fructus Gardeniae Jasminoidis	Gadernia Jasminoides Ellis	> 500

MIC: Minimum inhibitory concentration.

15 min. No. 11 tube was used as a growth control (broth with bacterial inoculum, no extract) and No. 12 tube was used as a sterility control (broth only). All tubes were cultured in a shaking incubator containing 5% O_2, 10% CO_2, and 85% N_2. At the same time, 10 μL bacterial suspension from No. 11 tube was diluted quickly with 10 mL 0.9% sterile physiological saline at 1:1000, then 100 μL 0.9% sterile physiological saline was transferred onto the surface of three Campylobacter plates [control of diarrheal diseases in China Research, Shanghai Regent Supply; each liter containing bio-polyone (10 g), bio-lysat (10 g), bio-myotone (3 g), corn starch (1 g), sodium chloride (5 g), agar (13.5 g), pH 7.3, autoclaved at 121℃ for 15 min] containing 5% (v/v) of sterile defibrinated sheep blood (Guangzhou Ruite Ltd., China), cultured at 37℃ in a jar system (Refrigerating Machine Factory, Yiwu City, Zhejiang Province, China) containing 5% O_2, 10% CO_2, and 85% N_2 to verify the absence of contamination and calculate the colonies. Susceptibility test for other medicinal plants was performed in duplicate.

RESULTS

After incubation, the tubes were visually examined to determine whether the *H. pylori* strains grew. *H. pylori* strains in No. 11 tube grew well and no bacterial growth was observed in No. 12 tube. The colonies in 3 agar plates grew well with an average number of about 50. The lowest concentration (highest dilution) of extract that inhibited the visible growth of *H. pylori* strains (no turbidity) was defined as MIC. For further confirmation, 10 μL of bacterial suspension from the clearly visible tubes was diluted quickly with 10 mL 0.9% sterile physiological saline at 1:1000, then 100 μL 0.9% sterile physiological saline was transferred onto the surface of three Campylobacter plates and cultured for 3 d. When the average number of colonies in the 3 agar plates was less than 5 or no colony was found, the MIC was considered less than or equal to the concentration. When the growth of *H. pylori* strains occurred in all dilutions containing the extract, the MIC was considered greater than the highest concentration. When no growth of *H. pylori* strains occurred in any concentration tested, the MIC was considered less than the lowest concentration. When a tube with visible growth of *H. pylori* strains, e.g. growth at 500, 250 and 62.5 mg/mL, but not at 125 mg/mL, was called a skipped tube and ignored. Growth of *H. pylori* strains in isolated tubes indicated contamination, the test should be repeated. The results are listed in Table 1. The MIC of Rhizoma Coptidis, Radix Scutellariae and Radix Isatidis was less than 7.8 mg/mL. The MIC of Floslonicerae Japonicae and Cortex Fraxini was less than 15.6 mg/mL. The MIC of Herba Violae cum Radce, Cortex Phellodendri and Foliun Isatidis, Herba Taraxaci Mongolici cum Radice was less than 31.2 mg/mL. The MIC of Rhizoma Rhei, Radix Sophorae Tonkinensis, Radix Gentianae Scabrae, Frutus Terminaliae Chebulae was less than 62.5 mg/mL. The MIC of Radix Sophorae Subprostratae, Herba Scutellariae Barbatae, Herba Houttuyniae Cordatae and Rhizoma Smilacis Glabrae was less than 125 mg/mL. The MIC of Fructus Arctii Lappae, Flos Chrysanthemum Morifolii, Herba Whiteflower Patrinia Herb, Radix Thichosanthis and Fructus Brucae Javanicae was less than 250 mg/mL. The MIC of Calculus Bovis, Frucifictio Lasiosphaerae, Folium Periliac Frutescentis, Radix Bupleuri, Caulis Lonicerae, Radix Sophorae Flavscentis was less than 500 mg/mL, and the MIC of other medicinal herbs was greater than 500 mg/mL. Authority books, such as Pharmacopoeia of People's Republic of China (Committee of National Pharmacopoeia, 2005 edition), Chinese Herbal Medicine (Gong-Wang Liu, Li-Ya Gao, 2000, Hua Xia Publishing House), Modern Clinical Chinese Herbal Medicines (Dong Kun-Shan, Wang Xiu-Qin, Dong Yi-Fan, 2001, Chinese Traditional Medicine Press), acclaimed that most selected medicinal plants have an activity against microscopic organisms, including various Gram-negative or -positive bacteria, fungi, viruses or parasites. In fact, many of them are the constituents of Chinese patent medicines used in treatment of stomach discomfort-related diseases. More importantly, most of them demonstrate a significant anti-*H. pylori* activity.

DISCUSSION

In this study, the MIC in 50 traditional Chinese herbal medicines was detected. Although a considerable variety of plants showing an antimicrobial activity have also been reported in other studies[7-19], variation of MIC still exists due to the bioassay methods employed in different studies, the sources and age of the plants, the solvent used for extraction, and *H. pylori* strains. The susceptibility of *H. pylori SS1* to water extracts was examined and screened in this study with broth dilution diffusion, a quantitative assay method, which is less time-consuming and less labor-intensive than agar dilution method, and cheaper than *E* test. All selected herbal medicines are the commonly used traditional Chinese herbal medicines prescribed by physicians of traditional Chinese medicine. Some plants are even recommended as a dietetic therapy for health preserving, such as Radix Isatidis, Herba Houttuyniae Cordatae. More importantly, all the selected herbal medicines have a same standard from the TonRen Corporation. Although the susceptibility of only an isolated *H. pylori* strain to such medicines was tested, the susceptibility of other clinical strains to these medicines should also be tested.

In this study, the water extract from Rhizoma Coptidis, Radix Scutellariae and Radix Isatidis had a stronger anti-*H. pylori* activity than that from other plants, indicating that the three plants can be used as useful sources for the synthesis of novel drugs against *H. pylori*. Traditional medical practitioners and biomedical specialists play an important role in pharmacodynamics and pharmacokinetics research. In order to find scientific evidence and rationalize the utility and efficacy of traditional Chinese medicines, they have tried to extract and analyze the active compounds of medicinal plants with various biomedical analytical techniques and assay methods, and evaluated their antibacterial and anti-inflammatory mechanism in animal experiments.

Rhizoma Coptidis contains berberine. Several protoberberine alkaloids of berberine, palmatine, coptisine and aporphinoid alkaloid of magnoflorine have been confirmed to be the major pharmacologically active constituents, and these alkaloids demonstrate a significant antimicrobial activity against a variety of organisms including bacteria, viruses, fungi, protozoans, helminthes, and Chlamydia[22,23]. The pharmacological antibacterial activity of the 3 berberine alkaloids is berberine > coptisine > palmatine[24]. An animal experiment suggested that the total alkaloid is a potent protective agent against *H. pylori* LPS which induces gastric mucosal inflammation[25].

Radix Scutellariae contains over 30 kinds of flavonoid, such as baicalin, baicalein, wogonin, wogonin7-glucuronide, oroxylin A, and oroxylin A 7-O-glucuronide[26]. Active flavonoids, including baicalin, baicalein, wogonin, and wogonoside, have a variety of pharmacological activities, such as anti-inflammation, free radical scavenging and anti-oxidation[27], and antibacterial action[28]. All active flavonoids exert their anti-inflammatory effect mainly by inhibiting the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (*COX-2*) gene expression[29]. It was

reported that Radix Scutellariae inhibits LPS-induced production of proinflammatory mediators, including NO, IL-3, IL-6, IL-10, IL-12p40, IL-17, IP-10, KC, and VEGF in mouse macrophages[30].

Radix Isatidis is also officially documented. Organic acids, including syringic acid, 2-amino-benzoic acid, salicylic acid and benzoic acid among the main chemical active components, have been segregated and purified as a crystal[31]. Some authors have even tested the potency sequence of the 4 organic acids (syringic acid > 2-amino-benzoic acid > salicylic acid > benzoic acid)[32]. Furthermore, the 4 organic acids share a basic molecular structure, and the number, position and type of their functional groups on phenyl ring have great impacts on antibacterial activities. Extracts from Radix Isatidis can decrease the production of inflammatory mediators, such as nitricoxide, prostaglandin E2, and pro-inflammatory cytokines[33].

Traditional Chinese medicines have a long history, due to their effectiveness and relatively low toxicity, and herbal medicines have drawn more and more attention during the past decades. Chemical compositions of Rhizoma Coptidis, Radix Scutellariae and Radix Isatidis have been extensively studied, some of which can act on *H. pylori* LPS and inhibit the production of proinflammatory mediators. In our study, water extracts from the medicinal plants demonstrated a strong anti-*H. pylori* activity, and a wide range of phytochemistry materials from medicinal plants could reduce the inflammatory response, indicating that the 3 herbal drugs can be used as anti-inflammatory or antibacterial agents. However, the strong *in vitro* anti-*H. pylori* activity of these water extracts does not necessarily imply that they have a strong *in vivo* anti-*H. pylori* activity. On the other hand, some of these plants may be more potent *in vivo* due to metabolic transformation of their components into highly active intermediates. However, further study is needed to confirm the effect of Rhizoma Coptidis, Radix Scutellariae, Radix Isatidis and other traditional Chinese medicines on alimentary tract diseases due to *H. pylori* infection.

It is well known that human beings are the main reservoir of *H. pylori*. World Health Organization pointed out that most subjects infected with *H. pylori* have no clinical symptoms, peptic ulceration and superficial chronic gastritis, but peptic ulcer, ulcer complications, and progression to gastric cancer will occur in approximately 17%, 4.25% and 1% of *H. pylori*-infected subjects, respectively[34]. Besides, extragastric diseases involving the cardiovascular, hepatobiliary, dermatological, immunological, hematological systems[35] are also related with *H. pylori* infection. Moreover, since *H. pylori* was isolated from human dental plaque[36], *H. pylori* has been detected in oral cavity, suggesting that oral cavity diseases such as halitosis, glossitis, burning mouth syndrome, recurrent aphthous stomatitis, dental caries, are related with oral *H. pylori* infection. Anand *et al*[37] reported that the prevalence of *H. pylori* is higher in dental plaque of patients with gastric *H. pylori* infection than in that of patients without gastric *H. pylori*. It has been shown that patients with poor oral hygiene have the most frequent recurrence of gastric *H. pylori* infection[38]. Oral cavity is a potential reservoir of *H. pylori* and oral *H. pylori* may influence the relapse of gastric *H. pylori* infection. It was reported that *H. pylori* in dental plaque is hardly eradicated by triple therapy[39,40], suggesting that oral antibiotics have almost no effect on *H. pylori* in oral cavity.

With the better recognition of *H. pylori*, more diseases have been found to be related to *H. pylori*. Since oral cavity, as a residence of *H. pylori*, is as important as stomach, prevention and treatment of oral *H. pylori* infection should be put on the agenda. Antibiotics have been the main drugs against *H. pylori* since the bacterium was discovered. Further study is needed to solve the problems such as drug resistance, poor patient compliance, undesirable side effects and the significant cost of combination therapy. Traditional Chinese medicines have shown their advantages over Western drugs, including a lower price, a low toxicity and less adverse reactions.

It is exciting that Rhizoma Coptidis, Radix Scutellariae, Radix isatidis and other herbs with a strong anti-*H. pylori* activity may provide the potential sources of new drugs, thus reducing the morbidity of oral cavity diseases and improving the eradication rate and relapse of gastric *H. pylori* infection.

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COMMENTS

Background

Many diseases are related to *Helicobacter pylori* (*H. pylori*) infection. Although antibiotics can eradicate gastric *H. pylori*, antibiotics treatment can lead the problems, such as drug resistance, poor patient compliance and undesirable side effects. It has been reported that some herbal medicines have an anti-*H. pylori* activity. The herbal medicine resources are rich in China with a long history of practicing traditional Chinese medicine. However, few studies are available on the anti-*H. pylori* activity of herbal medicines. Herbal medicines may be potential sources of new drugs.

Research frontiers

Human beings are the main reservoir of *H. pylori*. World Health Organization estimates indicate that *H. pylori* infection is closely related with gastric and extragastric diseases involving the cardiovascular, hepatobiliary, dermatological, immunological, and hematological systems. *H. pylori* has been detected in oral cavity. Oral cavity diseases such as halitosis, glossitis, burning mouth syndrome, recurrent aphthous stomatitis, dental caries, may be related with *H. pylori* infection.

Innovations and breakthroughs

In vitro susceptibility test was performed for water extract from 50 selected traditional Chinese herbal medicines and their anti-*H. pylori* activity was evaluated according their MIC values. The active compounds of Rhizoma Coptidis, Radix Scutellariae and Radix Isatidis, were detected and their anti-*H. pylori* activity was analyzed.

Applications

Since Rhizoma Coptidis, Radix Scutellariae and Radix Isatidis have a strong anti-*H. pylori* activity, with a low toxicity, a low price and less adverse reactions, they can be used in preventing and treating gastric and/or oral *H. pylori* infection.

Peer review

This study described the strong anti-*H. pylori* activity of Rhizoma Coptidis, Radix Scutellariae and Radix Isatidis, thus adding some novel herbal medicines for preventing and treating gastric and/or oral *H. pylori infection*.

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BRIEF ARTICLE

Genetic variations in the *SMAD4* gene and gastric cancer susceptibility

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Abstract

AIM: To explore the association between mothers against decapentaplegic homolog 4 (*SMAD4*) gene polymorphisms and gastric cancer risk.

METHODS: Five tagging single nucleotide polymorphisms (tSNPs) in the *SMAD4* gene were selected and genotyped in 322 gastric cancer cases and 351 cancer-free controls in a Chinese population by using the polymerase chain reactionrestriction fragment length polymorphism method. Immunohistochemistry was used to examine SMAD4 protein expression in 10 normal gastric tissues adjacent to tumors.

RESULTS: In the single-locus analysis, two significantly decreased risk polymorphisms for gastric cancer were observed: the SNP3 rs17663887 TC genotype (adjusted odds ratio = 0.38, 95% confidence interval: 0.21-0.71), compared with the wild-type TT genotype and the SNP5 rs12456284 GG genotype (0.31, 0.16-0.60), and with the wild-type AA genotype. In the combined analyses of these two tSNPs, the combined genotypes with 2-3 protective alleles (SNP3 C and SNP5 G allele) had a significantly decreased risk of gastric cancer (0.28, 0.16-0.49) than those with 0-1 protective allele. Furthermore, individuals with 0-1 protective allele had significantly decreased SMAD4 protein expression levels in the normal tissues adjacent to tumors than those with 2-3 protective alleles (P = 0.025).

CONCLUSION: These results suggest that genetic variants in the *SMAD4* gene play a protective role in gastric cancer in a Chinese population.

Key words: Mothers against decapentaplegic homolog 4; Genetic variation; Gastric tumor; Molecular epidemiology

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INTRODUCTION

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death worldwide; about 934 000 new cases were diagnosed and approximately 700 000 people died of the disease in 2002[1]. The incidence of gastric cancer varies within countries. In China, it was predicted that, in 2005, 300 000 deaths and 400 000 new cases from gastric cancer, which ranks it as the third most common cancer[2]. Epidemiological studies have identified many risk factors for gastric cancer, such as *Helicobacter pylori* (*H. pylori*) infection, low fiber intake, and tobacco smoking[3,4]. However, only a fraction of individuals exposed to these factors develop gastric cancer during their lifetime, which suggests that genetic susceptibility plays an important role in gastric carcinogenesis.

Transforming growth factor (TGF)-β signaling is one of the most important tumor suppressor pathways. Mothers against decapentaplegic homolog (SMAD) proteins serve as crucial components of TGF-β signaling, which negatively regulates cell growth and promotes apoptosis of epithelial cells. According to the specific functions, Smads can be classified into the receptor-regulated Smads (R-Smads: Smad 1, 2, 3, 5 and 8), inhibitory Smads (anti-Smads: Smad 6 and 7), and the common mediator Smads (Co-Smads: Smad 4), which is apparently common to all of the ligand-specific Smad pathways and plays a central role in TGF-β signaling[5]. In 1996, *SMAD4* was identified as a candidate tumor suppressor gene[6].

The loss of SMAD4 expression is a common feature of most human malignancies, including gastric cancer[7-11]. In 1997, Powell *et al*[10] firstly reported inactivation of SMAD4 in gastric carcinoma. Then, Xiangming *et al*[12] further demonstrated that the reduced expression of SMAD4 was 75.1% in advanced gastric cancer. Wang *et al*[13] has found that the loss of SMAD4, especially loss of nuclear SMAD4 expression, is involved in gastric cancer progression. A more recent study by Leng *et al*[14] has shown that SMAD4 expression in gastric cancer tissue is dramatically lower than that in peri-tumoral tissue. *In vitro* and *in vivo* studies have shown that mutations in *SMAD4* play a significant role in SMAD4 inactivation. For example, mutation-related loss of SMAD4 is most prevalent in pancreatic and colorectal cancer[8,15]. *SMAD4* mutations have also been observed in seminoma[16] and head and neck cancer[17], which give rise to the complete loss of SMAD4. Notably, germline mutations in *SMAD4* are found in > 50% of patients with familial juvenile polyposis syndrome[18,19], which predisposes individuals to develop gastrointestinal cancer. Although mutations in *SMAD4* are not seen frequently in gastric cancer (2.9%)[10], the gene is highly polymorphic in the dbSNP database.

Given the role of SMAD4 in tumor suppression, we hypothesized that genetic variants in the *SMAD4* gene are associated with the risk of gastric cancer. In the present study, five tagging single nucleotide polymorphisms (tSNPs) were selected to evaluate the association between these common genetic variants in *SMAD4* gene and risk of gastric cancer in our ongoing, hospital-based, case-control study in a Chinese population.

MATERIALS AND METHODS

Study subjects

The study included 322 gastric cancer patients and 351 cancer-free controls. All subjects were recruited from an ongoing study that started in March 2006. The detailed inclusion criteria have been described previously[20]. The participation rate of cases was about 95%. The cancer-free controls were genetically unrelated to the cases, had no individual history of cancer, and were recruited from the hospital where they were seeking health care or undergoing routine health examination. All the 351 control subjects were matched with the cases by age (± 5 years) and sex. Informed consent was obtained from each of the eligible subjects before recruitment. A questionnaire was used to obtain demographic and risk factor information about the study subjects. For gastric cancer patients, the clinicopathological variables, including tumor site, tumor histotype, invasion, and lymph node status, were obtained from the medical records of patients. The classification criteria of the clinicopathological variables were previously reported[20]. The response rate of the eligible controls was about 85%. Those subjects who smoked daily for > 1 year were defined as regular smokers. Individuals who consumed one or more alcoholic drinks per week for at least 1 year were considered regular drinkers. The research protocol was approved by the institutional review board of Nanjing Medical University.

SNP selection and genotyping

The *SMAD4* gene, which is 49.5-kb long and is located on chromosome 18q21.1, contains 13 exons and 12 introns. There have been at least 197 SNPs reported in the dbSNP database. Based on the HapMap database (http://www.hapmap.org/) (from chr1846807425 to 46868845), tSNPs were selected from common variants (minor allele frequency > 0.10) in the Han Chinese in Beijing (CHB) population sample. As a result, six tSNPs were selected using a pairwise Tagger method[21] with an r^2 cutoff value of 0.8 to capture all the common SNPs in *SMAD4* and the mean r^2 was 0.980. The genotype frequencies of the SNPs can be influenced by population differences and sample sizes[22,23], therefore, we genotyped these six tSNPs in 100 Chinese control subjects. Of these, one was not in agreement with the Hardy-Weinberg equilibrium (HWE) ($P < 0.01$). Thus, this tSNP was not included in further analyses. The rs number and relative position of selected five tSNPs are shown in Table 1.

The selected tSNPs were genotyped in all 673 subjects by the polymerase chain reaction (PCR)-restriction fragment length polymorphism method. The tSNPs information, primers, and restriction enzymes are all listed in

Table 1 Information on five genotyped tagging single nucleotide polymorphisms in the *SMAD4* gene

SNP No.	SNP ID	Location	MAF			*P*[2]	*P* for HWE[3]	Genotyping rate (%)
			Database[1]	Cases	Controls			
1	rs12958604	Intron 2	0.446	0.452	0.450	0.956	0.893	99.1
2	rs10502913	Intron 2	0.363	0.280	0.305	0.308	0.117	99.4
3	rs17663887	Intron 9	0.056	0.023	0.057	< 0.001	0.258	100.0
4	rs9304407	Intron 11	0.411	0.458	0.423	0.206	0.291	100.0
5	rs12456284	3′-UTR	0.405	0.289	0.350	0.017	0.231	100.0

[1]Minor allele frequency (MAF) for Han Chinese in Beijing (CHB) population in the HapMap database (http://www.hapmap.org); [2]*P* value for the allele distribution difference between the cases and controls; [3]Hardy-Weinberg equilibrium (HWE) *P* value in the control group. SNP: Single nucleotide polymorphism; 3′-UTR: 3′-untranslated region.

Supplementary Table 1. The genotype analysis was done by two persons independently in a blind fashion. About 1% of PCR products were randomly selected and confirmed by sequencing (data not shown), and > 10% of the samples were randomly selected for repeated genotyping. The results were 100% concordant.

Immunohistochemical staining and evaluation

Immunohistochemical study was performed on the 10 normal gastric tissues adjacent to tumors. Immunohistochemical staining was performed by using the Boster SABC (rabbit IgG)-POD Kit (Wuhan, China) according to the manufacturer's instructions. After deparaffinization and rehydration, the sections were microwaved for 10 min for antigen retrieval and then washed in PBS. Sections were incubated with normal goat serum for 30 min to block nonspecific antibody binding. The primary antibody anti-Smad4 (1:100; ab40759; Abcam Ltd., Hong Kong, China) was used to incubate sections overnight at 4℃, followed by three successive rinses with PBS, and incubation with secondary antibody for an additional 20 min. After rinsing, tissue sections were incubated with streptavidin-biotin-peroxidase (SABC) (Boster) for 20 min at room temperature. Slides were washed and visualized using 3,3'-diaminobenzidine. Slides were counterstained with hematoxylin, dehydrated, and mounted with balsam for examination.

A positive reaction was indicated by a reddish brown precipitate in the cytoplasm. Specifically, the percentage of positive cells was divided into five grades (percentage cores): (0) ≤ 5%; (1) 6%-25%; (2) 26%-50%; (3) 51%-75%; and (4) > 75%. Intensity of staining was divided into four grades (intensity scores): (0) no staining; (1) light brown; (2) brown; and (3) dark brown. SMAD4 staining positivity was determined by the formula: overall scores = percentage score × intensity score. Overall score of ≤ 3 was defined as negative, > 3 but ≤ 6 as weakly positive, and > 6 as strongly positive[24].

Statistical analysis

The χ^2 test was used to compare the differences in frequency distributions of selected demographic variables, smoking status, alcohol use, as well as each allele and genotype of the *SMAD4* polymorphisms between the cases and controls. The difference between *SMAD4* genotypes and clinicopathological characteristics was assessed by χ^2 test. The crude and adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were obtained to assess the association between the *SMAD4* polymorphisms and gastric cancer risk using, unconditional univariate and multivariate logistic regression models. The multivariate adjustment included the age, sex, tobacco smoking, and alcohol use. HWE of the genotype distribution among control groups was tested by a goodness-of-fit χ^2 test. The combined genotypes data were further stratified by subgroups of the age, sex, smoking status, and alcohol use. The Mann-Whitney *U* test was used to compare the SMAD4 expression levels between individuals with 0-1 protective allele and 2-3 protective alleles. All tests were performed with SAS software (version 9.1.3; SAS Institute, Inc., Cary, NC, USA) with two sides, unless indicated otherwise. $P < 0.05$ was considered statistically significant.

RESULTS

The characteristics of the study population are shown in Table 2. There was no significant difference in the distribution of age ($P = 0.354$), sex ($P = 0.516$), or alcohol use ($P = 0.846$) between the case and control subjects. However, there were more regular smokers among the cases (43.8%) than among the controls (35.0%) ($P = 0.020$). Furthermore, there were 149 (48.1%) and 161 (51.9%) patients with cardia and non-cardia gastric cancer, respectively. The histological types were 162 (52.3%) intestinal and 148 (47.7%) diffuse type gastric cancer; positive lymph nodes were identified in 148 (47.1%) cases. For depth of tumor infiltration, 80 (25.8%), 68 (22.0%), 112 (36.1%) and 50 (16.1%) cases were T1, T2, T3 and T4, respectively.

The primary information of the five tSNPs in CHB patients is shown in Table 1. The observed genotype frequencies of the five tSNPs among the control subjects were all in agreement with HWE (all $P > 0.05$). The allele frequencies of the genotyped tSNPs in the controls were consistent with those of the International HapMap Project database for CHB. The single SNP allele analysis indicated that the allele frequencies of two tSNPs, SNP3 rs17663887 and SNP5 rs12456284, were significantly different between the cases and controls ($P < 0.001$ for SNP3 rs17663887, and $P = 0.017$ for SNP5 rs12456284).

The genotype frequencies of these five tSNPs and

Table 2 Frequency distributions of selected variables between gastric cancer cases and cancer-free controls *n* (%)

Variables	Cases (*n* = 322)	Controls (*n* = 351)	*P*[1]
Age (yr)			
< 60	129 (40.1)	153 (43.6)	0.354
⩾ 60	193 (59.9)	198 (56.4)	
Sex			
Male	215 (66.8)	226 (64.4)	0.516
Female	107 (33.2)	125 (35.6)	
Smoking status			
Never	181 (56.2)	228 (65.0)	0.020
Regular	141 (43.8)	123 (35.0)	
Drinking status			
Never	217 (67.4)	239 (68.1)	0.846
Regular	105 (32.6)	112 (31.9)	
Tumor site[2]			
Cardia	149 (48.1)		
Non-cardia	161 (51.9)		
Histological types[2]			
Intestinal	162 (52.3)		
Diffuse	148 (47.7)		
Depth of tumor infiltration[2]			
T1	80 (25.8)		
T2	68 (22.0)		
T3	112 (36.1)		
T4	50 (16.1)		
Lymph node metastasis[2]			
Negative	166 (52.9)		
Positive	148 (47.1)		

[1]Two-sided χ^2 test for the frequency distribution of selected variables between gastric cancer cases and cancer-free controls; [2]No. of subjects in cases (n = 310 for tumor site, n = 310 for histological types, n = 310 for depth of tumor infiltration, and n = 314 for lymph node metastasis) were less than the total number (n = 322) because some information was not obtained.

their associations with gastric cancer risk are summarized in Table 3. The single locus analysis revealed that the genotype frequencies of two tSNPs, SNP3 rs17663887 and SNP5 rs12456284, were significantly different between the cases and controls ($P < 0.001$ for SNP3 and $P = 0.003$ for SNP5, respectively). Multivariate logistic regression analyses indicated that the variant TC genotype of SNP3 was associated with a significantly decreased risk of gastric cancer compared with the wild-type TT genotype (adjusted OR = 0.38, 95% CI: 0.21-0.71). For the SNP5, compared with the wild-type AA genotype, the variant GG genotype was associated with a statistically significantly decreased risk of gastric cancer (adjusted OR = 0.31, 95% CI: 0.16-0.60) (Table 3).

Considering the potential interactions of the tSNPs on risk of gastric cancer, we combined these two tSNPs based on the numbers of the protective alleles (i.e. SNP3 C and SNP5 G alleles). As shown in Table 4, the combined genotypes with zero and one protective allele were more common (0.429 and 0.518, respectively) and that with two and three protective alleles was less common (0.053 and 0.000, respectively) among the cases than the controls (0.362, 0.423, 0.154 and 0.011, respectively), and these differences were statistically significant ($P < 0.001$). When these combined genotypes were dichotomized into two groups (i.e. 0-1 *vs* 2-3 protective alleles), their distributions differed significantly between the cases and controls ($P < 0.001$).

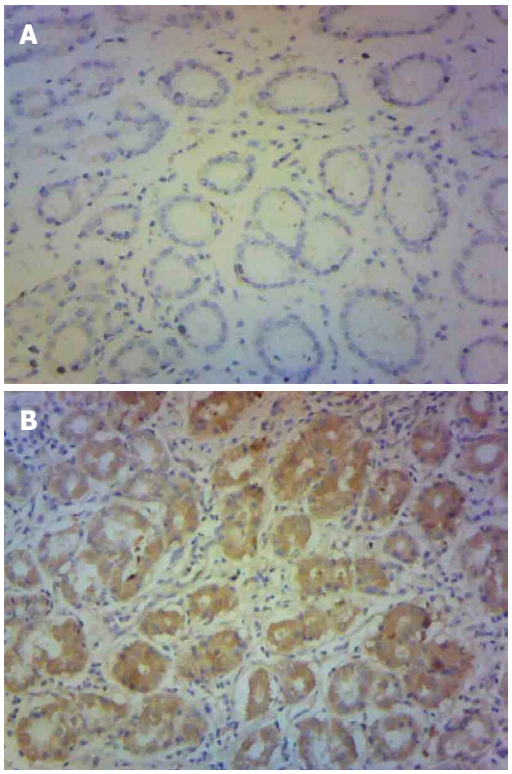

Figure 1 Immunohistochemical staining for SMAD4 in gastric tissues adjacent to tumor. HE, original magnification, 100 ×. A: Individuals with *SMAD4* 0 or 1 protective allele; B: Individuals with *SMAD4* 2 or 3 protective alleles.

In the association analyses, we found that the individuals with 2-3 protective alleles had a significantly decreased risk of gastric cancer (adjusted OR = 0.28, 95% CI: 0.16-0.49) than those with 0-1 protective allele (Table 4). Further stratification analysis showed the same results as the main protective effect among subgroups of age, sex, smoking status, and drinking status (data not shown). However, no statistical evidence was observed for interactions between the combined genotypes and the variables (i.e. age, sex, tobacco smoking, and alcohol use) (data not shown).

Based on the results of the genetic association studies of the *SMAD4* combined genotype (0-1 *vs* 2-3 protective alleles) and gastric cancer, the SMAD4 protein expression of gastric cancer patients with 0-1 or 2-3 protective alleles was analyzed using immunohistochemistry. Four of the 10 patients had 2-3 protective alleles and six had 0-1 protective allele. SMAD4 was mainly expressed in the cytoplasm. Individuals with 0-1 protective allele had significantly decreased SMAD4 expression compared with those with 2-3 protective alleles ($P = 0.025$) (Figure 1).

To explore whether genetic variation in SMAD4 is associated with clinicopathological characteristics and disease progression, we performed additional stratified analysis of association between *SMAD4* combined variant genotypes and risk of gastric cancer by the tumor sites (cardia and non-cardia), histological types (intestinal and diffuse), tumor infiltration (T1-T4), and lymph node metastasis (negative and positive). However, no significant association was observed (data not shown).

Table 3 Genotype distributions of the *SMAD4* tSNPs in gastric cancer cases and controls and risk estimates *n* (%)

SNP No.	SNP ID	Genotypes	Cases (*n* = 322)	Controls (*n* = 351)[1]	*P* value (2df)[2]	Crude OR (95% CI)	Adjusted OR (95% CI)[3]
1	rs12958604	AA	99 (30.8)	106 (30.5)	0.961	1.00	1.00
		AG	155 (48.1)	171 (49.1)		0.97 (0.68-1.38)	0.98 (0.69-1.39)
		GG	68 (21.1)	71 (20.4)		1.03 (0.67-1.59)	1.01 (0.66-1.56)
2	rs10502913	GG	164 (51.0)	162 (46.4)	0.505	1.00	1.00
		GA	136 (42.2)	161 (46.1)		0.83 (0.61-1.14)	0.86 (0.63-1.18)
		AA	22 (6.8)	26 (7.5)		0.84 (0.46-1.54)	0.81 (0.44-1.49)
3	rs17663887	TT	307 (95.3)	311 (88.6)	< 0.001	1.00	1.00
		TC	15 (4.7)	40 (11.4)		0.38 (0.21-0.70)	0.38 (0.21-0.71)
4	rs9304407	GG	85 (26.4)	112 (31.9)	0.291	1.00	1.00
		GC	179 (55.6)	181 (51.6)		1.30 (0.92-1.85)	1.32 (0.93-1.87)
		CC	58 (18.0)	58 (16.5)		1.32 (0.83-2.09)	1.29 (0.81-2.05)
5	rs12456284	AA	149 (46.3)	143 (40.7)	0.003	1.00	1.00
		AG	160 (49.7)	170 (48.4)		0.90 (0.66-1.24)	0.90 (0.66-1.24)
		GG	13 (4.0)	38 (10.8)		0.33 (0.17-0.64)	0.31 (0.16-0.60)

[1]No. of subjects in controls (*n* = 348 for SNP1, *n* = 349 for SNP2) were less than the total number (*n* = 351) because some DNA could not be genotyped; [2]Two-sided χ^2 test for the frequency distribution; [3]Adjusted for age, sex, smoking status, and alcohol use. SNP: Single nucleotide polymorphism; OR: Odds ratio; CI: Confidence interval.

Table 4 Frequency distributions of the combined genotypes of *SMAD4* SNP3 and SNP5 between gastric cancers and controls *n* (%)

No. variant (protective) alleles of the combined genotypes[1]	Cases (*n* = 322)	Controls (*n* = 351)	*P*[2]	Adjusted OR (95% CI)[3]
0	138 (42.9)	127 (36.2)	< 0.001	
1	167 (51.8)	166 (42.3)		
2	17 (5.3)	54 (15.4)		
3	0 (0.0)	4 (1.1)		
Dichotomized groups				
0-1	305 (94.7)	293 (83.5)	< 0.001	1.00
2-3	17 (5.3)	58 (16.5)		0.28 (0.16–0.49)

[1]0-3 represent the number of variants within the combined genotypes (0 = no variant and 1-3 = 1-3 variants); the variant (protective) alleles used for the calculation were the SNP3 C and SNP5 G alleles; [2]Two-sided χ^2 test for the frequency distribution; [3]Odds ratios (ORs) were obtained from a logistic regression model with adjustment for age, sex, smoking status, and alcohol use. SNP: Single nucleotide polymorphism; CI: Confidence interval.

DISCUSSION

In this case-control study of gastric cancer, we investigated the associations of five tSNPs located in the intron (SNP1-4) and 3'-untranslated region (3'-UTR) (SNP5) of the tumor suppressor gene *SMAD4* with risk of gastric cancer in a Chinese population. Among these five tSNPs, we found that two variant genotypes (SNP3 TC and SNP5 GG) were associated with a significantly decreased risk of gastric cancer. When the protective alleles (SNP3 C and SNP5 G alleles) were evaluated together, we found that individuals with 2-3 alleles had a significantly decreased risk of gastric cancer compared with those with 0-1 protective allele. Furthermore, individuals with 0-1 protective allele had a significantly decreased SMAD4 protein expression level in normal tissues adjacent to tumors compared with those with 2-3 protective alleles. To the best of our knowledge, no published studies have investigated the role of *SMAD4* polymorphisms in gastric cancer.

It has been shown that the loss of SMAD4 is a common feature of most human malignancies, and is associated with cancer progression[13]. Smad4 complete knockout mice can generate tumors throughout the gastrointestinal tract[25,26]. Experimental data also suggest that SMAD4 participates in immunosuppression. Absence of SMAD4 expression in thymic epithelial cells leads to functional change, and the number of early T-lineage progenitors is markedly reduced[27]. Selective loss of SMAD4 in T cells also leads to epithelial cancers throughout the gastrointestinal tract in mice[28]. SMAD4 appears to be a key regulatory protein of the SMAD4-dependent signaling in tumor carcinogenesis.

In the present study, we found an association of *SMAD4* tSNPs with risk of gastric cancer. Moreover, SMAD4 protein expression levels were significantly different between individuals with 0-1 and 2-3 protective alleles. Although the underlying mechanism by which the mutated intron allele or 3'-UTR allele in the gene is associated with cancer risk remains elusive, there are two possible explanations. First, the mutant C allele of SNP3 rs17663887 that is located in intron 9 might produce/alter *cis* elements that allow/alter binding of transcription factors and thereby change *SMAD4* expression. Using the Alibaba program (http://www.gene-regulation.com/cgi-bin/pub/programs/alibaba2), we found the transcription factors are altered when the SNP3 T allele mutates to C allele [i.e. T allele: C/EBPα (CCAAT/enhancer binding protein α), HNF-3 (fork-head homolog 3), and AP-1

(activator protein 1); C allele: C/EBPβ (CCAAT/enhancer binding protein β) and HSF (heat shock factor)]. Second, the mutant G allele of SNP5 rs12456284 that is located in the 3'-UTR might influence the potential miRNA binding and ultimately influence *SMAD4* expression. Our immunohistochemistry assay partly supported these assumptions *in vivo*. However, the experiment needs to be done in normal gastric tissues and with larger sample sizes.

In this study, we found that *SMAD4* polymorphisms might jointly provide protection against gastric cancer risk; individuals with 2-3 protective alleles had a significantly decreased risk of gastric cancer compared with those with 0-1 protective allele. This putatively supports the notion that a single polymorphism only contributes a modest effect and the combined variants of a gene might provide a more comprehensive evaluation of genetic susceptibility in candidate genes with low penetration. To date, few published epidemiological studies have investigated the associations between *SMAD4* polymorphisms and human cancer. Only one case-control study has reported an association between the *SMAD4* tSNPs and testicular germ cell tumor susceptibility in the US Servicemen's Testicular Tumor Environmental and Endocrine Determinants Study[29]. Although they also selected the two tSNPs in the *SMAD4* gene (i.e. rs9304407 and rs12456284) in their study, no association was found.

Kim *et al*[30] have reported that loss of SMAD4 protein expression is significantly associated with intestinal type gastric cancer. Later, they further reported that expression of SMAD4 was significantly lower in diffuse than intestinal-type gastric cancer[31]. Nevertheless, our analysis failed to find an association of *SMAD4* polymorphisms with tumor histological types. Besides, Xiangming *et al*[12] and Kim *et al*[30] have reported that reduced expression of SMAD4 is related to the depth of tumor invasion. Our results did not find any correlation between the polymorphisms and tumor infiltration of gastric cancer. This could be attributable to different ethnicity and our relatively small sample size. Larger studies with different ethnic populations are needed.

Several limitations in our study need to be addressed. (1) The study design was hospital-based, which could have had inherent limitations that introduced selection bias, compared with population-based or cohort studies. However, the allele frequency in control subjects is close to that reported in the HapMap database for the CHB population; (2) We did not obtain enough information on *H. pylori* infection, and future studies with such information are needed; and (3) The relatively small sample size of 322 cases and 351 controls in the present study might not be large enough to identify significant gene-environment interactions, although we had > 85% power to detect an OR of $\geqslant$ 1.6 and $\leqslant$ 0.6, with an exposure frequency of 30% under the current sample size.

In conclusion, we found two tSNPs within the *SMAD4* gene that were associated with a decreased risk of gastric cancer in a Chinese population. This is believed to be the first report of *SMAD4* polymorphisms and gastric cancer, therefore, additional larger investigations and functional studies with more detailed environmental exposure data are warranted to validate these findings.

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COMMENTS

Background

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death worldwide. Epidemiological studies have identified many risk factors for gastric cancer that are involved in genetic susceptibility.

Research frontiers

Mothers against decapentaplegic homolog 4 (SMAD4) is a central mediator of the transforming growth factor β signaling pathway, which acts as a tumor suppressor in numerous cancers. The relationship between *SMAD4* gene polymorphism and gastric cancer needs to be addressed.

Innovations and breakthroughs

This is believed to be the first study to examine the potential role of *SMAD4* genetic variants in the occurrence of gastric cancer in a Chinese population. Two tSNPs within the *SMAD4* gene were found to be associated with a decreased risk of gastric cancer. Protein expression assays also support the association study results.

Applications

These findings might be of value in the explanation of gastric carcinogenesis. The observations also could be used as for further investigation of SMAD4 and gastric cancer.

Peer review

This paper is generally well prepared.

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BRIEF ARTICLE

Relationship between RGS5 expression and differentiation and angiogenesis of gastric carcinoma

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Abstract

AIM: To explore the regulator of G-protein signaling 5 (RGS5) expression in gastric carcinoma and its association with differentiation and microvascular density (MVD).

METHODS: Expression of RGS5 and CD34 were examined in 76 cases of gastric carcinoma, including 22 cases with lymph node metastasis and 54 cases without lymph node metastasis determined by immunohistochemistry (IHC). MVD was assessed using CD34 monoclonal antibody. The presence of RGS5 and CD34 was analyzed by IHC using the Envision technique.

RESULTS: The RGS5 expression in gastric carcinoma was positively correlated with the differentiation of the tumor (r = 0.345, P < 0.001), but not related with age, gender, tumor size, clinical stage and lymph node metastasis (P > 0.05). The average MVD in the group with lymph node metastasis was significantly higher than that in the group without lymph node metastasis (P < 0.05). RGS5 expression was negatively correlated with the average MVD (P < 0.05).

CONCLUSION: RGS5 expression level in gastric carcinoma is associated with the differentiation and MVD of the tumor, and may be used as an important parameter for determining the prognosis of gastric carcinoma patients.

Key words: RGS5 expression; Gastric carcinoma; Differentiation; Microvascular density

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INTRODUCTION

Gastric carcinoma is one of the most common malignant tumors, and lymph node metastasis is the most common and the earliest transfer channels. Regulator of G-protein signaling 5 (RGS5) is a member of the RGS superfamily and acts as a negative regulator of heterotrimeric G-protein-mediated signaling through G-protein-coupled recep-

tors (GPCRs)[1,2]. Recently, RGS5 has been found involved in tumor angiogenesis and metastasis[3-6]. We used immunohistochemical technique to examine the expression of RGS5 and CD34 in 76 cases of gastric carcinoma in order to explore the relationship between RGS5 expression and differentiation and angiogenesis of gastric carcinoma.

MATERIALS AND METHODS

Samples, histological examination and reagents

We studied 76 patients with gastric adenocarcinoma who underwent operation without radiotherapy and chemotherapy in the Nanjing General Hospital of Nanjing Military Command, from March 2005 to October 2009, including 46 men and 30 women, with an average age of 55.95 years. There were 42 stage Ⅰ, 12 stage Ⅱ and 22 stage Ⅲ cases; the tumors were highly differentiated in 22, moderately differentiated in 30 and poorly differentiated in 24 cases. Two senior pathologist reviewed the morphologic classification of the tumors according to the WHO specifications and evaluated the adequacy of biopsy specimens for further tests. Specimens were promptly fixed and embedded, and the slices were made with a thickness of 2-4 μm.

The antibody used for RGS5 was rabbit polyclonal (Sigma Inc., USA) at 1:80 dilution. The antibody used for CD34 was mouse monoclonal (Neo Markers Inc., USA) at 1:200 dilution. The diaminobenzidine tetrahydrochloride was obtained from DAKO Company.

Immunohistochemistry

The presence of RGS5 and CD34 was analyzed by Immunohistochemistry (IHC) using the Envision technique. Antigen retrieval was carried out by high temperature and pressure cooking of the slices in 15 mL EDTA. The slices were rinsed in phosphate buffered solution (PBS) (0.01 mol/L, pH 7.4) for three times, incubated with the primary antibody overnight at 4℃ and washed again in PBS for three times. They were then incubated with the anti-rabbit and mouse horse radish peroxidase polymer reagent for 12 min at room temperature, and washed in PBS three times as above. The reaction product was developed using diaminobenzidine tetrahydrochloride. Finally, the slices were counterstained with hematoxylin, dehydrated and mounted in resinous mountant. Negative controls with PBS (0.01 mol/L, pH 7.4) replacing the primary antibody were also included.

Any brown cytoplasmic staining of cells was taken as positive expression for RGS5. The tissue sections were screened at a high power (× 200) and five areas with the most intense expression were selected. Briefly, a mean percentage of positive cells was determined in at least five areas (× 200) and assigned to one of the following five categories: < 5% (-); 5%-25% (+); 25%-50% (2+); 50%-75% (3+); and ≥ 75% (4+). A mean percentage of positive cells < 50% was considered as having low expression, and that ≥ 50% was considered as having high expression. Microvascular density (MVD) was assessed using CD34 monoclonal antibody. Any brown cytoplasmic or membranous staining of vascular endothelial cells was taken as positive expression for CD34. The tissue sections were screened at a low power (× 40) and three areas with the most intense neovascularization were selected. Microvessel counting was performed at a high power (× 400) in these areas.

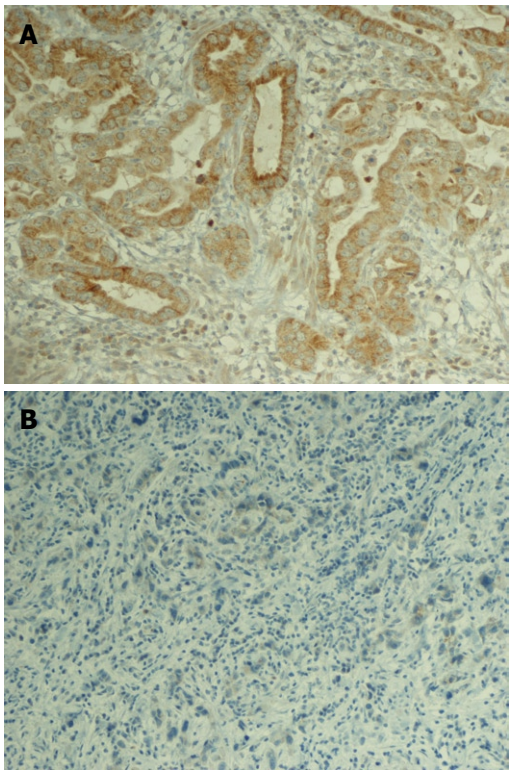

Figure 1 Expression of regulator of G-protein signaling 5 (× 200). A: High regulator of G-protein signaling 5 (RGS5) expression in highly differentiated gastric adenocarcinoma; B: Low RGS5 expression in poorly differentiated gastric adenocarcinoma.

Statistical analysis

Statistical analyses were performed using the Software Packages for Social Science 13.0 for Windows (SPSS, Inc, Chicago, IL, USA). Associations of RGS5 expression with clinical parameters of patients were described by the Chi-square test. Fisher's exact test was also used when necessary. Relationship between expression of RGS5 and clinical parameters of patients was analyzed using the Spearman rank correlation analysis. Average MVD was calculated and analyzed using the independent-samples *T* test. *P* values < 0.05 were considered significant.

RESULTS

Expression of RGS5

RGS5 was mainly expressed in the cytoplasm of gastric carcinoma cells, and the strong positions were in the regions infiltrated by the tumor cells (Figure 1). The RGS5 expression level in gastric carcinoma was positively correlated with the differentiation ($r = 0.345$, $P < 0.001$, Table 1),

Table 1 Relationship between clinical parameters of patients and expression of regulator of G-protein signaling 5 in gastric carcinoma

Variables	*n*	+	+ +	+ + +	+ + + +	Spearman correlation	*P*
Differentiation						0.345	0.000
Low	24	16	4	4	0		
Moderate	30	4	8	12	6		
High	22	6	8	0	8		
Gender						-0.219	0.059
Male	46	12	14	8	12		
Female	30	14	6	8	2		
Lymph node metastasis						0.082	0.343
Positive	22	8	8	2	4		
Negative	54	18	12	14	10		
Tumor size (cm)						-0.053	0.766
≤ 4	58	20	14	12	12		
> 4	18	6	6	4	2		
Age (yr)						0.122	0.197
≤ 55	36	16	6	8	6		
> 55	40	10	14	8	8		
Clinical stages						-0.192	0.184
Ⅰ	42	12	8	12	10		
Ⅱ	12	6	4	2	0		
Ⅲ	22	8	8	2	4		

Table 2 Relationship between microvascular density and clinical parameters of patients and expression of regulator of G-protein signaling 5 in gastric carcinoma

Variables	*n*	MVD (mean ± SD)	*P*
Gender			
Male	46	11.08 ± 7.87	0.221
Female	30	16.07 ± 13.99	
Lymph node metastasis			
Positive	22	19.17 ± 12.03	0.014
Negative	54	10.29 ± 9.04	
Tumor size (cm)			
≤ 4	58	13.59 ± 12.06	0.548
> 4	18	11.27 ± 5.87	
Age (yr)			
≤ 55	36	11.61 ± 10.23	0.481
> 55	40	14.05 ± 11.17	
Expression of RGS5			
High	30	9.15 ± 7.16	0.023
Low	46	16.75 ± 12.37	

MVD: Microvascular density; RGS5: Regulator of G-protein signaling 5.

but not correlated with age, gender, tumor size, clinical stages and lymph node metastasis ($P > 0.05$, Table 1).

CD34 was expressed in the cytoplasm or membrane of vascular endothelial cells. The staining of cells was uniform. And the areas with intense microvascularization were the regions infiltrated by the tumor cells (Figure 2). The average MVD in the group with lymph node metastasis group was significantly higher than that in the group without lymph node metastasis ($P < 0.05$, Table 2), but not correlated with age, gender and tumor size ($P > 0.05$, Table 2).

Relationship between RGS5 expression and MVD in gastric carcinoma

The average MVD in high RGS5 expression group was significantly lower than that in low RGS5 expression group ($P < 0.05$, Table 2, Figure 2).

DISCUSSION

G protein-coupled biological processes are important for an ever-increasing number of human diseases[7]. RGS5 is a member of the RGS superfamily, and is involved in a number of processes of diseases, such as atherosclerosis[8]. In normal tissues, RGS5 was found to be highly up-regulated in PDGFR-β^+ pericytes and played an important role in developmental processes of blood vessels[9-12]. Recently, it has been indicated that RGS5 expresses in the early stages of blood vessel maturation and regulates the development of vascular pericytes[13-23]. However, there have been fewer studies about RGS5 expression in tumors. Chen *et al*[3] and Furuya *et al*[4] discovered that RGS5 highly expressed in vascular pericytes of both hepatocellular carcinoma and renal cell carcinoma. In addition, tumor metastasis depends on the newborn blood vessels, and RGS5 was found involved in tumor angiogenesis[24]. However, the function of RGS5 in development or angiogenesis of gastric carcinoma is still unclear.

We used IHC method for the first time to examine the expression of RGS5 protein in gastric carcinoma. The results showed that expression of RGS5 protein varies in different gastric carcinoma patients, indicating that the function of RGS5 protein in occurrence of gastric carcinoma is manifold. Forty-six (61%) cases had low RGS5 expression, and most of them had poorly differentiated gastric carcinoma ($P < 0.001$). Tumor occurrence and development are associated with multiple genes, and RGS5 protein is only a part of the signal transduction pathway. RGS5 acts as a negative regulator of heterotrimeric G-protein-mediated signaling through GPCRs[1]. RGS5

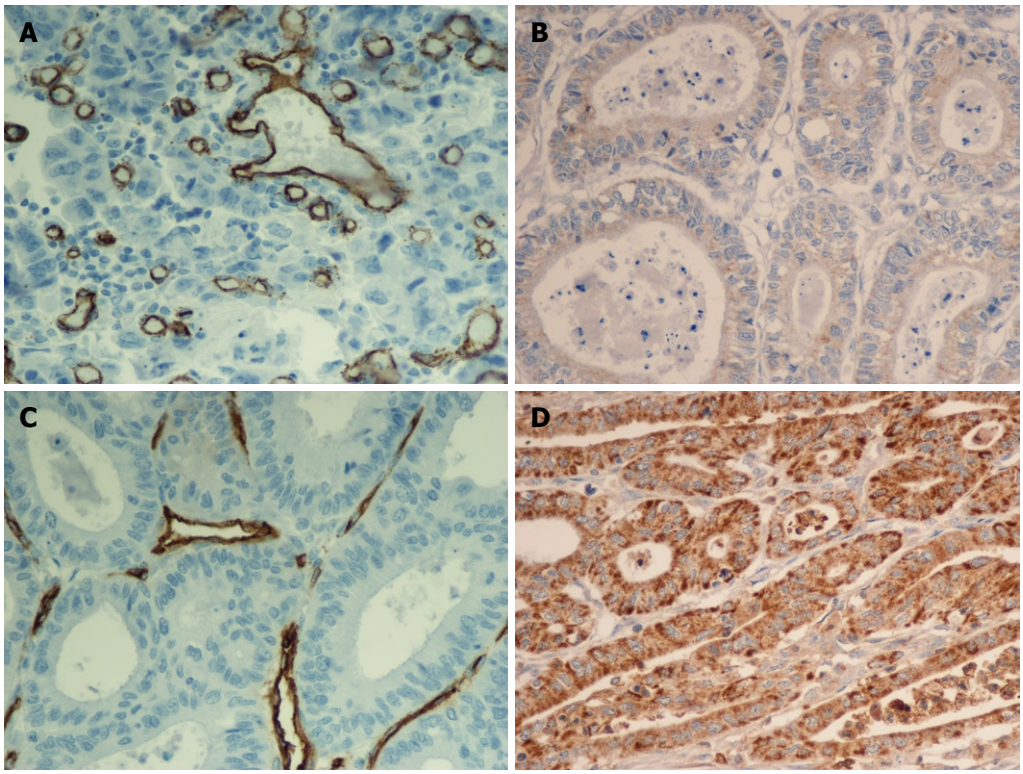

Figure 2 High microvascular density in gastric adenocarcinoma with low regulator of G-protein signaling 5 expression (A, B) and low microvascular density in gastric adenocarcinoma with high regulator of G-protein signaling 5 expression (C, D). Envision, × 400.

was found to be involved in tumor angiogenesis, and perhaps regulate tumor progress.

We found that the RGS5 expression level in gastric carcinoma was negatively correlated with the average MVD. The average MVD in the group with lymph node metastasis was significantly higher than that in the group without lymph node metastasis, but the RGS5 expression level in gastric carcinoma was not correlated with lymph node metastasis. This may be related to the number of samples and methods of detection. In addition, in the aspect of regulation of the tumor growth, we have found that the tumor blood vessels of RGS5 gene knockout mice tended to be mature and differentiated[25-28], indicating that expression of RGS5 is a factor of tumor blood vessel abnormalities and may play a certain role in regulating the invasion and metastasis of tumor cells.

In addition, RGS5 expression can be used to determine the prognosis of renal clear cell carcinoma together with a number of other indicators[8,29,30]. Our study suggested that RGS5 expression level in gastric carcinoma was significantly associated with the differentiation and MVD of the tumor, also indicating the trend of disease development. Many researches have confirmed that the differentiation, MVD and some other clinical pathological parameters are important factors for the prognosis of gastric carcinoma. Therefore, RGS5 protein may be an effective indicator for evaluating the malignant degree of gastric carcinoma.

However, there have been fewer studies about the relationship between RGS5 and tumors reported in literature. The function of RGS5 in tumor development or tumor angiogenesis needs to be further studied.

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COMMENTS

Background

G protein-coupled biological processes are important for an ever-increasing number of human diseases. Regulator of G-protein signaling 5 (RGS5) is a member of the RGS superfamily and acts as a negative regulator of heterotrimeric G-protein-mediated signaling through G-protein-coupled receptors. Recently, RGS5 has been found involved in tumor angiogenesis and metastasis.

Research frontiers

High RGS5 expression has been found in the vascular pericytes of hepatocellular carcinoma and renal cell carcinoma. In addition, tumor metastasis depends on the newborn blood vessels, and RGS5 is involved in tumor angiogenesis. The expression of RGS5 is a factor of tumor blood vessel abnormalities and may play a certain role in regulating invasion and metastasis of tumor cells.

Innovations and breakthroughs

The authors used immunohistochemical method for the first time to examine the expression of RGS5 protein in gastric carcinoma.

Applications

There have been fewer studies about the relationship between RGS5 and tumors. The function of RGS5 in tumor development or tumor angiogenesis needs to be further studied.

Peer review

In this manuscript, Wang *et al* examined the expression of RGS5 and its relationship with differentiation and microvascular density (MVD) in a panel of gastric tumors. They found that RGS5 expression is associated with differentiation,

and negatively correlated with average MVD. These findings have not been described and are potentially important. The quality of representative pictures is high.

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CASE REPORT

Erythrocytic transglutaminase inhibition hemolysis at presentation of celiac disease

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Abstract

Celiac disease (CD) is a common autoimmune condition. Previously it was considered to be a rare childhood disorder, but is actually considered a relatively common condition, present at any age, which may have multiple complications and manifestations. Hematological disorders of the disease are not uncommon. Among these disorders, the most frequently reported are anemias as a result of iron deficiency, often associated with folate and/or B12 deficiency. Anemias caused by hemolysis are very rarely reported in celiac patients. An 11-year-old girl with a previous uneventful medical history presented with severe hemolytic anemia. Hemolysis was Coombs negative, accompanied by inappropriate low reticulocyte count, despite exaggerated bone marrow hyperplasia of the erythroid precursors which showed normal maturation. Serology for recent infections, including Epstein-Barr virus, parvovirus B19, cytomegalovirus and mycoplasma, were all negative. Levels of serum IgA, IgG and IgM, were all within normal ranges for age. Screening for anti-DNA, antinuclear, antineutrophil cytoplasmic, antimicrosomal, antithyroglobulin, and antimitochondrial antibodies and lupus anticoagulants, was negative. She was also negative for human immunodeficiency virus. Conventional therapy with corticosteroids and intravenous immunoglobulin failed. CD was serendipitously discovered upon screening for anti-tissue transglutaminase autoantibodies. The disease was confirmed by biopsy of the small intestine mucosa. The patient recovered with gluten-free diet. A unique case of CD is presented. CD should be serologically screened in each patient with Coombs negative "immune" hemolytic anemia, particularly if accompanied by "reticulocytopenia". A new hemolytic mechanism and very speculative explanation for "reticulocytopenia" are discussed.

Key words: Celiac disease; Tissue transglutaminase; Antibodies; Hemolytic anemia; Gluten free diet

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INTRODUCTION

Celiac disease (CD) is a common autoimmune condition, induced by the intake of prolamines, alcohol soluble proteins, rich in glutamine and proline present in wheat, barley and rye, in genetically susceptible persons. Histologically, the disease produces a spectrum of upper small intestinal mucosa changes, ranging from an increase in

the number of intraepithelial lymphocytes to mucosal remodeling with crypt hyperplasia and flattening of the villi. Contrary to common belief this disorder is a systemic protean disease, rather than merely a pure digestive dysfunction. Previously, it was considered to be a rare childhood disorder, but is actually considered a relatively common condition, present at any age, and may have multiple complications and manifestations[1]. Among these, hematological disorders at presentation of the disease, and/or as manifestations during the course of the disease are not uncommon. The most frequent are anemias, including iron-deficiency anemia often associated with folate and/or B_{12} deficiency[2]. Other, less common hematological disorders seen in CD are leucopenia/neutropenia, thrombocytopenia, thrombocytosis, and vitamin K deficiency, manifested as coagulopathy and/or thromboembolism[2]. Hemolytic anemia, as an integral part of the clinical picture of CD, is extremely rare. We describe a child whose CD presented with severe hemolytic anemia with inappropriate reticulocytosis. A new mechanism of hemolysis, specific for CD and a very speculative explanation for "reticulocytopenia" are discussed.

CASE REPORT

An 11-year-old girl, with no previous gastrointestinal or other complaints, presented with acute, severe hemolytic anemia. This was preceded by slight flu-like illness of 2 d duration. On admission she was found to be pale, with mild icteric discoloration of the sclera. She was above the 25th percentile for weight and height. The spleen was slightly enlarged. Initial laboratory testing disclosed an elevated sedimentation rate of 100/146, hemoglobin 4.3 g/L, red blood cell count 1.5×10^{12}/L, and white blood cell count 9.8×10^{6}/L. A peripheral blood smear showed normal white cells and differential, normochromic and normocytic red cells. Neither abnormal red cells, nor normoblasts were seen. The platelet count was 355×10^{9}/L, mean corpuscular volume 83 fL, reticulocyte percentage 3.0%, total bilirubin level 46 μmol/L (normal range 1.7-20.0 μmol/L), direct bilirubin level 13 μmol/L (normal range 1.7-8.6 μmol/L), lactate dehydrogenase 980 U/L, slightly elevated serum transaminases activities (alanine transaminase 67 U/L, aspartate transaminase 72 U/L), and almost undetectable haptoglobin level (< 0.07 g/L). Bone marrow examination revealed hyperplasia of the erythroid precursors with normal maturation. Serology for recent infections, including Epstein-Barr virus, parvovirus B19, cytomegalovirus and mycoplasma, were all negative. A direct antiglobulin test (DAT) with anti-IgG, anti-IgM and anti-C3b was negative. An indirect antiglobulin test was also negative. The ceruloplasmin level was 46.1 g/L. Levels of serum IgA, IgG and IgM, were all within the normal ranges for age. Screening for anti-DNA, antinuclear, antineutrophil cytoplasmic, antimicrosomal, antithyroglobulin, and antimitochondrial antibodies and lupus anticoagulants, was negative. She was also negative for human immunodeficiency virus.

A diagnosis of idiopathic DAT-negative autoimmune hemolytic anemia (AIHA) was established. For the first 7 d, in expectation of spontaneous recovery, she was treated with transfusions of red packed cells only. Hemolysis was so severe that she had to receive at least 500 mL of red packed cells daily to maintain the hemoglobin level just above 50 g/L. On the 7th day, 3 mg/kg per day of prednisone was started with 1 g/kg of intravenous immunoglobulin (IVIG) on 2 consecutive days. She did not improve during the following 14 d. Then we started to taper prednisone, and serendipitously[3] the patient's blood was sampled for anti-tissue transglutaminase antibodies (anti-tTGAbs) and a gluten-free diet (GFD) was introduced. A gradual improvement ensued. The need for blood transfusion started to decrease 7 d after introduction of the GFD and a steady increase in hemoglobin level followed, accompanied by exaggerated reticulocytosis (> 20%). Ten days after blood sampling for anti-tTGAbs testing, we were informed that the anti-tTGAb titer was elevated (45.8 U/mL, normal < 8 U/mL). Thereafter a peroral intestinal enterobiopsy was performed. CD grade Ⅲ was confirmed. Now, 5 years later, on a GFD, she has normal development, normal blood counts, and normal anti-tTGAbs titer.

DISCUSSION

There is no doubt that our patient has CD, which was previously unrecognized. The patient had an uneventful medical history, until a severe, life-threatening hematological disorder appeared. There is also no doubt that the nature of the hematological disorder was anemia caused by a hemolytic mechanism. Until recently we thought that the patient had classical AIHA with negative DAT. AIHA is very rare event in CD and in the general population as well[4]. AIHA is not even included in the hematological spectrum of CD[2], despite the existence of 4 well documented reports of patients having CD and AIHA, (2 had Evans syndrome)[5-7]. These patients, contrary to the presented case were: (1) symptomatic before CD was diagnosed; (2) their hemolysis was DAT positive suggesting that the hemolysis was IgG and/or C3b mediated; (3) hemolysis in these patients was accompanied by exaggerated reticulocytosis; and finally (4) each of these patients responded to corticosteroid treatment. Our patient was (1) asymptomatic before hemolysis appeared; (2) hemolysis was resistant to corticosteroids and IVIG; (3) DAT was negative, meaning that the hemolysis was not mediated by IgG, IgM or C3b; and finally (4) despite the large number of erythroid precursors in the patient's bone marrow, reticulocytes were only marginally elevated (3%).

In an attempt to explain these unusual phenomena in our patient, like The Three Princes[3], we set out on a trip, and kept making unexpected discoveries along our way through the medical literature. Thus, we reached the wonderful world of transglutaminases (TGs)[8], especially the world of tissue transglutaminase (tTG), and their "enemies", the anti-tTGAbs[9]. Anti-tTGAbs found in CD[9]

have special properties which may play a role in the disease, at both local and systemic levels, reflecting the role of tTG in many crucial biological processes. It was shown that anti-tTG-Abs from celiac patients inhibit the enzymatic activity of human tTG both *in vitro*, and *in situ*[10]. Very recent studies showed that *in vivo* targeting of tTG in CD also occurs in the form of *in situ* endomysial, reticulin, and jejunal subepithelial anti-tTGAbs binding[11]. Furthermore, the same study[11] showed IgA deposition on extracellular tTG in the liver, lymph nodes, and muscles indicating that the CD autoantigen is widely accessible to the intestinally-produced circulating autoantibodies throughout the body.

Finally, we were able to elucidate the mystery of our patient. Anti-tTGAbs were produced in her small intestine upon continuous exposure to gluten and distributed *via* the circulation throughout the body. They targeted the erythrocytic TG, so called band 4.2[12] and disabled its anchor function. As a consequence, the patient's erythrocytes became fragile and hemolysis ensued in the very hostile spleen environment (acidosis, hypoglycemia, slowed circulation).

This case should not be classified as classical IgG or C3b mediated AIHA. It should be rather considered as a "celiac type of immune hemolysis", because we believe it was mediated by autoantibodies, which were generated in the intestinal mucosa in gluten intolerance and exerted an anti-tTG activity. This kind of hemolysis could be designated as an enzymatic inhibition induced hemolysis. One could argue that the hemolysis in our patient could be IgA mediated. Unfortunately, at the time in Serbia kits for anti-IgA DAT were not available. Because our patient was not IgA deficient, it could be presumed that the patient's circulating anti-tTGAbs which belong to IgA, targeted the erythrocytic TG (band 4.2). One can also argue that our patient could have AIHA with a low titer of anti-erythrocytic antibodies to explain the negative Coombs test. Having in mind the severity of the hemolysis, we can argue with the question: can low anti-erythrocyte autoantibodies cause such severe hemolysis?

The failure of our patient to respond to corticosteroids and IVIG could be explained by the fact that the patient was fed, i.e. continuously challenged, with gluten and the production of anti-tTGAbs was unrestrained, until the introduction of a GFD.

Finally, we will try to explain the most mysterious phenomenon of our patient and that is the inappropriate low reticulocytes (3%), despite the exaggerated erythroid bone marrow hyperplasia which ensued as a physiologic answer to severe hemolysis.

Fibronectin is a glycoprotein associated with the extracellular matrix of many tissues[13] and is a major component of the interstitial matrix in the bone marrow[14]. Fibronectin is tightly bound to the surface of many cells, including bone marrow precursors. Migration of mature reticulocytes from the bone marrow into the circulation is associated with the loss of fibronectin adhesion from the marrow precursors (mature reticulocytes). This process is probably physiologically mediated by at least 2 bone marrow tTGs. The first one is cell surface tTG, a ubiquitously expressed, potent integrin-binding adhesion coreceptor involved in the binding of cells to fibronectin[15]. Its role would be retention of early erythroid marrow precursors in the bone marrow hematopoietic nests, until their full maturation. After that another tTG, an extracellular tTG, would modulate fibronectin adhesion affinity. This tTG switches fibronectin adhesion from the mature reticulocyte membrane toward the extracellular matrix, *via* tight cross-linking of fibronectin with extracellular matrix proteins[16]. Thus, reticulocytes are liberated and migrate into the circulation.

We suggest that, in our patient, anti-tTGAbs targeted this extracellular bone marrow tTG, and fibronectin adhesion affinity could not be switched, which resulted in trapping of reticulocytes in the bone marrow. This is a very speculative explanation. We cannot offer any other explanation for the low reticulocyte count in our patient, despite the bone marrow erythroid hyperplasia.

This case represents one of the many atypical presentations of CD, and the first one whose CD started with hemolytic anemia. CD should be serologically screened in every patient with DAT-negative immune hemolytic anemia, particularly if accompanied by "reticulocytopenia". This case also confirms the value of antibody screening in early diagnosis of CD. Finally, we believe that we have probably described a new mechanism of hemolysis, which we would designate as an "enzymatic inhibition hemolysis".

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

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Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
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Organization as author

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

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

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Contents

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Contents

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EDITORIAL

Non-steroidal anti-inflammatory drugs: What is the actual risk of liver damage?

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) constitute a family of drugs, which taken as a group, represents one of the most frequently prescribed around the world. Thus, not surprisingly NSAIDs, along with anti-infectious agents, list on the top for causes of Drug-Induced Liver Injury (DILI). The incidence of liver disease induced by NSAIDs reported in clinical studies is fairly uniform ranging from 0.29/100 000 [95% confidence interval (CI): 0.17-051] to 9/100 000 (95% CI: 6-15). However, compared with these results, a higher risk of liver-related hospitalizations was reported (3-23 per 100 000 patients). NSAIDs exhibit a broad spectrum of liver damage ranging from asymptomatic, transient, hyper-transaminasemia to fulminant hepatic failure. However, under-reporting of asymptomatic, mild cases, as well as of those with transient liver-tests alteration, in conjunction with reports non-compliant with pharmacovigilance criteria to ascertain DILI and flawed epidemiological studies, jeopardize the chance to ascertain the actual risk of NSAIDs hepatotoxicity. Several NSAIDs, namely bromfenac, ibufenac and benoxaprofen, have been withdrawn from the market due to hepatotoxicity; others like nimesulide were never marketed in some countries and withdrawn in others. Indeed, the controversy concerning the actual risk of severe liver disease persists within NSAIDs research. The present work intends (1) to provide a critical analysis of the dissimilar results currently available in the literature concerning the epidemiology of NSAIDS hepatotoxicity; and (2) to review the risk of hepatotoxicity for each one of the most commonly employed compounds of the NSAIDs family, based on past and recently published data.

Key words: Non-steroidal anti-inflammatory drugs; Side effects; Fulminant hepatic failure; Cholestasis; Liver damage; Liver injury; Hepatitis; Hepatotoxicity

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are consumed massively worldwide and, along with antimicrobial agents, are the most frequent causes of drug-induced liver injury (DILI)[1-3]. Indeed, roughly 10% of total drug-induced hepatotoxicity is NSAIDs related. Recent data from England indicates that the relative frequency of NSAIDs prescription has changed, i.e. a 4% decline in the consumption of diclofenac and a parallel 5% increase in the use of naproxen are probably due to an increase in the awareness of both gastrointestinal and cardiovascular serious adverse events[4]. The current policy concerning the use of NSAIDs recommends that patients take "the lowest effective dose for the shortest duration" needed to control symptoms. In this particular

setting evidence indicates that naproxen is associated with a lower thrombotic risk than coxibs, and that ibuprofen has a good thrombotic safety profile for daily doses of up to 1200 mg[5]. Noticeably, 6% of the US population consumes at least one of these products in a month. Information regarding ibuprofen indicates that 24% is sold as over the counter medication[6].

The paradox is that even when it has been known historically and widely accepted that the use of these NSAIDs is associated with a low rate of hepatic adverse events, it is severe liver damage that is the main adverse event through which these drugs are eventually withdrawn from the market[7]. On the other hand, 50% of fulminant hepatic failure (FHF) in the USA is due to hepatotoxicity[8]. Antimicrobial drugs are the most often involved[9].

Hepatotoxicity is more frequently encountered in post-marketing studies or even after, once the drug has already been launched due to the fact that premarketing recruits a relatively low number of patients, frequently insufficient to fully judge the true incidence of hepatotoxicity[10]. One of the most representative examples of this situation is that of bromfenac-related severe liver damage. Bromfenac was withdrawn from the market in 1998 as a result of multiple reports of FHF within the year of its approval (1 case for every 10000 exposed individuals)[11,12]. Frequent severe liver damage associated with nimesulide had also been reported[13]. Although nimesulide is currently approved in 50 countries worldwide, national health authorities of several countries have withdrawn nimesulide from the market and others have never approved it[14-16]. EMEA (European Medicines Evaluation Agency) currently recommends a restricted length of treatment (15 d) and a maximal drug dosage (100 mg/d) for nimesulide therapy, which in addition should be avoided in children.

Interestingly, NSAIDs-induced hepatotoxicity may be associated with liver injury six to nine times more frequently in patients who are taking other potentially hepatotoxic medications concomitantly. (i.e. amoxicillin-clavulanic acid, proton pump inhibitor, phenobarbitol, isoniazid)[17,18].

According to a recent study, hepatic steatosis and metabolic syndrome might predispose to an NSAIDs-induced liver toxicity[19], a similar situation to that already proven for methotrexate and halothane.

Finally, chronic alcohol abuse during treatment with paracetamol was associated with an increase in liver injury induced by acetaminophen including those patients who were taking doses not higher than 4 g daily[20].

Our attempt in the present work is two-fold (1) to analyze the clinical impact regarding severity of NSAIDs hepatotoxicity based on a critical analysis of the conflicting epidemiological approaches currently available in the literature; and (2) to review the individual expected risk of hepatotoxicity for NSAIDs most commonly employed in clinical practice based on past and recently published data.

EPIDEMIOLOGY

Epidemiological studies evaluating severe NSAID liver toxicity take into consideration hospitalization and death of the exposed population. One important pitfall to demonstrate the risk of liver damage induced by NSAIDs resides in the epidemiological study design.

Generally speaking, the strengths of randomized control trials (RCT) are the close patient follow up, the comparison with a control group and an accepted design in order to prove specific therapeutic actions. However, RCT frequently represent just a sample of the general population seldom truly representative, particularly as evidence of the incidence of hepatotoxicity[6]. In addition, trials frequently fail to be informative concerning populations under 18 years of age because this group of individuals is usually underrepresented when not openly excluded[21]. On the other hand, data regarding incidence of liver toxicity from cohort, case-control studies and trials are not free from bias. Environmental factors, alcohol abuse, viral infections and metabolic factors coexist with DILI constituting confounding factors**.** Likewise, retrospective studies have important drawbacks such as researcher unawareness of concurrent drug intake other than the study drug. Other important limiting factors affect both pre- and post-marketing hepatotoxicity studies. Among these is the fact that mild and reversible cases are underreported. The other frequent source of error is the lack of a reliable "denominator" (defined as the number of patients potentially exposed) for calculations. We may also consider an additional source of underestimation of hepatotoxicity which is the report lag - the delay between the occurrence of adverse events and case communication. The latter should be taken into account when the alert is associated with severe liver damage. The incidence of liver disease induced by NSAIDs reported in most studies is fairly uniform ranging from 1 to 9 cases per 100000 persons exposed[21,22]. In spite of these results, epidemiologists analyzing the real risk of liver disease induced by different NSAIDs usually face difficulties, such as different study designs, different populations (ethnic groups, age and sex) and adjustment variable control methods that add complexity to the data analysis.

Traversa and co-workers analyzed a retrospective study from 1997 through 2001 in a region of Italy (850000 inhabitants). About 2 million prescriptions corresponding to NSAIDs-treated patients through a 5-year period follow-up were analyzed[23].

One of the main conclusions drawn from this study is that the risk of NSAIDs induced hepatotoxicity is very small (if the number of prescriptions is taken as the denominator, the incidence of liver injury was 1.7 per 100000 exposed individuals). In contrast, a higher rate of hepatotoxicity was observed among people older than 75 years old (5.7-fold increased risk of liver disease when compared with people under 45 years). Interestingly, it was observed that nimesulide showed both a slightly higher incidence of liver damage and a higher hospitalization rate than that observed with the other NSAIDs (33 per 100000 patient-years *vs* 22 per 100000 patient-years respectively). The authors were unable to find severe liver damage and deaths related to NSAIDs. While the positive features of this study were the high number of patients

Table 1 Results of population-based studies showing liver toxicity induced by non-steroidal anti-inflammatory drugs

Authors	Study design	Incidence rate of hepatotoxicity (ALT ⩾ 3 ULN) per 100 000 patient-years (95% CI)
García Rodríguez *et al*[22], 1992	Retrospective cohort study, cross over design	9/100 000 (6-15)
Traversa *et al*[23], 2003	Retrospective cohort	1.4/100 000 (1.0-2.1)
de Abajo *et al*[24], 2004	Retrospective population-based case-control study	2.4/100 000 (2.0-2.8)
Rubenstein *et al*[26], 2004	Systematic review (hospitalization or death)	3.1 to 23.4/100 000
Rostom *et al*[21], 2005	Systematic review	0.29/100 000 (0.17-0.51)
Laine *et al*[25], 2009	Long-term prospective trial	2.1/100 000 (1.9-2.3)

ALT: Alanine transaminase; ULN: Upper limit of normal; CI: Confidence interval.

enrolled and the extended follow-up, the major limitations were that the database monitoring system neither included the reason for prescription nor NSAIDs doses.

In contrast, a case-control study that included prescriptions of all market-approved NSAIDs highlighted diclofenac as the only drug associated with an increased risk of liver damage (95% confidence interval: 1.9-8.8)[24]. Laine and co-workers recently reported the largest prospective, randomized double-blind study comprising four times more patients than the largest previous trials. They evaluated the incidence of diclofenac-induced hepatotoxicity in 17 289 patients, showing that patients who suffered diclofenac-associated adverse drugs reactions seldom required hospital admission (23/100 000 patients). They also observed that symptoms of diclofenac-related liver disease developed either early or late after starting drug therapy[25]. The conclusions were: a low rate of occurrence of diclofenac-related admissions, and a very low rate of diclofenac-associated FHF. Indeed, only one patient required liver hospitalization for every 132 patients with aminotransferase > 3 × upper limit of normal (ULN).

Rostom and co-workers investigated bibliographic databases MEDLINE and EMBASE and public FDA archives in order to identify randomized controlled trials of diclofenac, naproxen, ibuprofen, celecoxib, rofecoxib, valdecoxib, or meloxicam in adults with osteoarthritis or rheumatoid arthritis. The authors analyzed aminotransferase elevations > 3 ULN, liver-related drug discontinuation, serious hepatic adverse events, liver-related hospitalizations, and liver-related deaths[21]. After analyzing 65 database articles and 67 FDA submitted studies, they concluded that diclofenac and rofecoxib had a higher level of transaminases both compared with placebo and with the other studied NSAIDs. Interestingly, none of these studies had a high rate of serious hepatic adverse events, hospitalizations or death.

The authors found only 1 hospital admission (naproxen) observed among 37 671 patients included in studies reporting hospitalization. This very low hospitalization rate represents 3 per 100 000 patients (0.5-15 per 100 000 patients). One patient died due to naproxen liver toxicity among 51 942 patients consuming NSAIDs, which in turn also represents a low death rate: 2 per 100 000 patients (0.3-11 per 100 000 patients).

These results are in concordance with those reported by Rubenstein and Laine who also analyzed several epidemiologic studies designed to determine the incidence and risk of serious liver-related NSAIDs toxicity[26]. Seven studies met the inclusion criteria proposed by the authors. They observed an incidence of liver toxicity associated with hospital admission ranging from 3.1-23.4/100 000 patient-years related to current use of NSAIDs, with an excess risk compared with past NSAIDs users of 4.8-8.6/100 000 patient-years of exposure. Moreover, these researchers documented zero mortality associated with NSAIDs when cumulative exposure of liver damage was analyzed in 396 392 patients/year.

Most of the information regarding the incidence and relative risk of hepatotoxicity associated with NSAIDs comes from cohort or case control studies and usually shows a low incidence of hepatotoxicity (Table 1).

Great efforts have been made to identify those clinical factors predictive of severe liver damage induced by drugs. Several years ago the FDA along with Representatives of Pharmaceutical Research and Manufacturers of America (PhRMA) and the American Association for the Study of Liver Disease (AASLD) constituted a working group to study how to minimize the risk of hepatotoxicity[27]. Despite valuable effort, expert consensus could not be achieved concerning: (1) biochemical markers of liver injury to applied used in pre-marketing studies; and (2) clinical parameters able to predict severe liver injury. Yet today we still continue using transaminases level higher than three times the ULN as a marker of significant hepatocellular injury[28].

CHARACTERISTICS OF NSAIDS INDUCED LIVER TOXICITY

The discovery of aspirin in 1946 followed by that of phenylbutazone was the beginning of the NSAIDs era. However, not until 1960 was indomethacin marketed. On the other hand, during the 1950s, ibuprofen was the second drug (along with aspirin) approved to be sold as over the counter medication. Interestingly, most of these substances were employed during the 60s, before the prostaglandin era.

The NSAIDs chemical classification recognizes four major groups of molecules: (1) carboxylic acids; (2) oxicams carboxamides; (3) sulphonanilides diaryl-substituted; and (4) pyrazole/furanones[29]. From the clinical stand point NSAIDs induced hepatotoxicity is associated with

Table 2 Liver-related hepatotoxicity induced by non-steroidal anti-inflammatory drugs

Drug	Pattern of liver damage	Proposed mechanism	Incidence
Aspirin	Acute and chronic hepatitis Reye's syndrome	Dose dependent > with high dose	Low
Diclofenac	Acute and chronic hepatitis Mixed damage and pure cholestasis	Metabolic Immunologic	Low
Sulindac	Acute hepatitis and mixed injury	Hypersensivity	Moderate
Ibuprofen	Acute hepatitis, ductopenia	Metabolic	Low
Naproxen	Cholestatic, mixed damage	Metabolic	Low
Coxibs	Acute hepatitis, mixed damage	Probably metabolic	Low
Oxicams	Acute hepatitis, massive and submassive necrosis, cholestasis and ductopenia	Metabolic	Low
Nimesulide	Acute hepatitis, pure cholestasis	Probably metabolic	Moderate

different patterns of clinical presentation, several mechanisms of liver damage and various pathological patterns.

We will only describe below the clinical characteristics of several NSAIDs that may be associated with a potential liver injury (Table 2).

Acetaminophen was not taken into account because most cases of liver damage are due to suicide attempts but a minority of cases are accidental and related to use of paracetamol as a therapeutic prescription. Acetaminophen-induced hepatotoxicity should be described separately and not within this topic.

ASPIRIN

Although liver toxicity induced by aspirin is considered to be dose-dependent, there is evidence that rheumatic patients may have predisposing conditions that may increase individual risk of liver damage. Hypoalbuminemia in patients with systemic lupus erythematosus and juvenile rheumatoid arthritis are two well documented risk factors for increased susceptibility to liver injury[30,31]. In addition, studies in rats have shown that aspirin hepatotoxicity is more common in animals with experimentally-induced rheumatoid arthritis compared to that observed in those without arthritis[32]. The clinical presentation of liver toxicity is often anicteric (jaundice at less than 5%) and transaminase levels correlate with serum salicylate levels (> 25 mg/100 mL)[33]. Focal nonspecific necrosis, hepatocellular degeneration and hydropic changes are commonly seen in liver histology[34]. Aspirin can also produce a mitochondrial dysfunction pattern that may lead to a liver free fatty acid accumulation and subsequently develop into a severe metabolic disorder associated with hepatic massive micro-steatosis. This syndrome, known as Reye's Syndrome, is characterized by metabolic acidosis, hepatic encephalopathy, hypoglycemia, coagulopathy and azotemia. Reye's disease induced by aspirin is a rarity since aspirin is currently avoided in pediatric patients and replaced by ibuprofen and paracetamol.

A recent experimental study in rats suggests that salicylic acid could trigger mitochondrial dysfunction causing a marked fall in intracellular ATP which in turn leads to a lethal hepatocellular injury mediated by a lipid peroxidation mechanism[35].

DICLOFENAC

Diclofenac is the most widely used NSAID in the world[36,37]. The vast majority of data related to hepatic reactions comes from retrospective studies. There were no more than 60 cases of diclofenac hepatotoxicity reported in the literature until Banks and co-workers in 1995 reported their analysis of 180 cases referred to FDA from 1988 through 1991. The authors observed evidence of liver disease in 85% of the patients within the first 6 mo after drug intake. Interestingly, a higher latency (after 6 mo) was observed in 12% of cases[38]. The long latency period observed in a large number of cases in addition to the absence of hypersensibility support a metabolic mechanism of hepatotoxicity. Jaundice was a very common sign present in 90 out of 120 patients. A total of 7 jaundiced patients died as a result of liver disease. In this analysis liver function tests (LFTs) showed a mixed (hepatocellular and cholestatic) pattern in 66% of cases, cholestatic in 8% and indeterminate in the remainder of the group. In contrast to Jick's point of view which suggests that hepatotoxicity induced by diclofenac is an uncommon event[39], the study by Banks proposed for the first time that diclofenac is a much more common cause of liver damage than so far suspected[38]. This concept was reinforced in a recent report from Laine who conducted the longest and largest liver-related diclofenac study so far ever reported [Multinational Etoricoxib and Diclofenac Arthritis Long-Term (MEDAL) program][25]. They conducted a prospective, randomized and double-blind study analyzing the frequency of diclofenac induced liver reactions in 17 289 patients. The authors randomized only those osteoarthritis or rheumatoid arthritis patients over 50 years of age who were going to receive diclofenac therapy (150 mg daily) or etoricoxib (60 or 90 mg daily). They also excluded cases with previous liver disease or more than 14 weekly alcohol drinks. Hypertransaminasemia > 3 × ULN was observed in over 3% of arthritic patients with a regular intake of diclofenac. It is interesting to point out that alanine transaminase (ALT) values higher than 10 × ULN were only identified in 0.5% of cases[25]. The incidence of diclofenac liver-related hospitalizations in this study was 16 per 100 000 patient-years. Through these results, the authors provide evidence that diclofenac is a very safe drug. The

small number of related hospitalizations (0.023%), and Hy's cases (transaminases > × 3 ULN and bilirubin > 2 ULN or a fatal outcome or liver transplantation) (0.012%) are the strongest evidence showing that diclofenac bears a low liver toxicity rate.

These data are in concordance with those observed by Traversa and co-workers showing ibuprofen and naproxen also displayed a low hepatotoxicity rate. However, in this latter study nimesulide had a higher hospitalization rate when compared with a past control cohort (33 per 100 000 patient-years)[23]. In a systematic analysis of several randomized studies, Rostom and co-workers also found a very low frequency of hospital admissions due to NSAIDs related-liver disease in 4261 patients (3 per 100 000 patient) and 0% when diclofenac was evaluated separately[21].

Diclofenac is a typical example of the combination of factors resulting in hepatotoxicity (e.g. drug metabolism, reactive metabolite formation and clearance) determining the actual development and the severity of liver damage. Moreover, diclofenac might produce liver injury through either metabolic idiosyncrasy or an immunological mechanism generated by drug adduct formation[8].

SULINDAC

Sulindac (SLD) is associated with an increased incidence of liver toxicity and serious hepatic reactions (5-10 times) when compared with other NSAIDs. Besides its recognized anti-inflammatory mechanism through the inhibition of cyclooxygenase (COX 1 and 2), SLD became very popular due to its antiapoptotic effect in colonic polyposis treatment[40]. SLD induced hepatotoxicity was more frequently encountered among people over 50 years old. Regarding the mechanism of hepatotoxicity, SLD associated DILI is one other example of a combination of factors as judged by the conflicting results present in the literature. Indeed, in a series of 91 documented cases reported to FDA a predominantly cholestatic pattern was present in 43% while hepatocellular-related changes were found in 25% of patients[41]. Moreover, a hypersensitivity mechanism of liver injury was present in 60% of cases. Zou *et al*[42] recently reported an interesting pilot study showing that co-treatment of SLD and lipopolysaccharide (LPS) caused liver injury in rats. In this context they also found a selectively clotting system activation and fibrinolytic system inhibition in rats treated with SLD/LPS. These changes were also associated with tisular hypoxia and fibrin clot deposit in the hepatic sinusoids[42]. Researchers suggest that these results may be extrapolated to humans with the disease. Furthermore, they hypothesize that hypoxia in the frame of the SLD/LPS association may underlie the idiosyncratic model where the sulfide metabolite probably plays a central role.

Another experimental study carried out by the same authors shows that tumor necrosis factor (TNF)-α augmented the cytotoxicity of SLD sulfide in primary hepatocytes and HepG2 cells. These results suggest that TNF-α can enhance SLD sulfide-induced hepatotoxicity, thereby contributing to liver injury in SLD/LPS-cotreated rats[43].

Regrettably the current coexistence of conflicting and heterogeneous results precludes us from reaching valid conclusions regarding SLD DILI.

IBUPROFEN

Ibuprofen has a recognized anti-inflammatory, analgesic and antipyretic property and is one of the most commonly NSAIDs used worldwide. It is characterized by a high safety profile and very low liver toxicity incidence. Along with paracetamol and aspirin, ibuprofen has become one of the largest ever selling over the counter drugs. It was first introduced to the UK market in 1969 and due to the low rate of gastrointestinal adverse events, it has ever since almost replaced aspirin, indometacin and phenylbutazone in arthritic patients[44]. A scarce number of hepatotoxicity reports involving ibuprofen were published, associated to both hepatocellular and cholestatic liver damage. Indeed, one of the latter cases was linked to vanishing bile duct syndrome[45,46]. It has also been suggested that ibuprofen may increase the risk of liver injury when administered to patients with chronic hepatitis C. An ibuprofen associated increase of transaminases > 5 × UNL was recently reported in three patients with chronic hepatitis C, eventually confirmed by re-challenge[47].

Traversa *et al*[23] in their cohort study that recruited thousands of patients receiving various NSAIDs confirmed that ibuprofen has a very low liver toxicity rate: Only two patients showed ibuprofen-associated liver injury (out of 126 cases that had NSAIDs). Despite the massive use of this drug worldwide, a low rate of ibuprofen liver toxicity along with a low incidence of gastrointestinal, renal and cardiovascular serious events characterizes the safety profile. This is probably based on the fact ibuprofen has a short plasma half life and does not form pathological metabolites.The absence of reports including ibuprofen induced liver injury in several studies and meta-analyses, strongly suggests that ibuprofen is an unlikely cause of liver disease[48-50].

COXIBS

Coxibs are NSAIDs designed to selectively inhibit COX-2. Lately, this group of drugs has been gaining worldwide popularity due to a much better gastrointestinal safety profile when compared with nonselective NSAIDs[25]. However, NSAIDs-induced cardiovascular adverse events have generated significant controversy[51,52]. Due to increased risk of myocardial infarction and arterial hypertension, rofecoxib was removed from the market by Merck in 2004[53]. Valdecoxib was also voluntarily discontinued by manufacturer Bextra, Pfizer Canada Inc and by FDA in 2005 because of severe allergic skin lesions including cases of Stevens-Johnson syndrome and also because of an increased risk of heart and stroke attack[54]. On the other hand, celecoxib continues to be marketed in many countries. Laine and coworkers recently published a comprehensive evaluation of the literature up to 2007

analyzing controlled trials, meta-analyses and reviews related to the safety profile of selective inhibitors of COX-2 in patients with osteoarthritis (OA)[55]. Their results are truly encouraging and show that coxibs have a therapeutic efficacy comparable to other NSAIDs and higher than that of acetaminophen. This was observed when patients with OA were treated for moderate to severe degree of pain. Through meta-analysis the authors documented that coxibs have a 74% lower risk of gastro-duodenal ulcer complications. In contrast, they found a double risk of myocardial infarction vs placebo and naproxen treated patients. There were no significant differences in cardiovascular risk between coxibs compared with non-naproxen NSAIDs. Despite these encouraging results, FDA reported an alert related to the increase of cardiovascular risk induced by coxibs.

Coxib-induced liver injury is an uncommon event and the annual reported incidence of hepatotoxicity is 1 in 100 000 exposed persons[56]. A long term study evaluating the safety profile of celecoxib in arthritics patients (Celecoxib Long-term Arthritis Safety Study-CLASS) found increased transaminase level in 0.6 % patients[57].

A rise of transaminase level was also reported and associated with rofecoxib (2%) and with higher doses of lumiracoxib (3%). In this study, a higher frequency of clinical hepatitis was found in patients receiving 400 mg of lumiracoxib when compared with ibuprofen and naproxen treated patients[58]. In another survey, lumiracoxib was linked to severe hepatocellular necrosis[59]. Health authorities from United Kingdom subsequently removed it from the market. Scottish authorities reported 20 cases of severe liver disease probably associated with lumiracoxib. FHF was documented in 14 out of 20 patients (two patients died and three patients needed liver transplantation). In addition, recent postmarketing reports from Australia of severe hepatic reactions with lumiracoxib at doses of at least 200 mg (two patients died and 2 underwent liver transplantation) led also to its withdrawal from the market in that country[60].

On the other hand, rofecoxib was associated with a low rate of hepatic reactions and an increase of ALT $\geqslant$ 3 × UNL was documented in 1.8/100 000 exposed persons[55], but despite this excellent safety profile, Yan and colleagues reported two well-documented cases of cholestatic hepatitis induced by rofecoxib[61]. The first patient showed a high level of alkaline phosphatase (APH) associated with hepatocellular injury in zone 1 whereas in the other case a significant increase in ALT associated with a minimum increase of APH was documented. In this latter patient mild liver damage in both acinar zones 1 and 3 was described. In contrast, four other published cases of rofecoxib induced liver toxicity were characterized by predominant cholestatic presentation[62,63].

To our knowledge no case of etoricoxib severe hepatotoxicity has ever been published. However, transaminase elevation (3 × ULN) has been reported in 1% of patients treated with etoricoxib for up to one year[21,64].The mechanism of liver damage induced by coxibs has not yet been completely elucidated. Kung and co-workers recently suggested that the bioactivation of lumiracoxib and its metabolite [4'-hydroxylumiracoxib (M5)] may produce GSH depletion, covalent binding to proteins and oxidative stress, that in turn may lead to liver injury[65].

Nevertheless, hepatotoxicity caused by non-selective NSAIDs has been more extensively studied. Furthermore, mitochondrial injury, cholestasis and oxidative stress induced by a reactive metabolite formation constitute the most conspicuous molecular reported disorders. An attractive hypothesis suggests that NSAIDs inhibition of COX-2, might cause liver damage through a prostaglandin (PGs) pathway. Regarding this concept the authors proposed that the inhibition of PGE2 could down regulate the antiapoptotic mitochondrial protein Bcl-2, which protects against bile acid induced apoptosis[66].

OXICAMS

Oxicams induced hepatotoxicity is an uncommon clinical situation. Piroxicam induced severe hepatocellular necrosis was the most frequent reported clinical pattern[35,67,68]. Uneventful recovery, death and need of liver transplantation have all been reported[69,70]. On the other hand, a clinical and histological pattern of hepatocellular plus cholestatic (mixed) injury was also associated with piroxicam in one single case. LFTs normalization was observed in this patient within 4 mo after starting the clinical disease[71], Interestingly, a case of severe intrahepatic cholestasis linked to a long period of piroxicam intake, was originally described 20 years ago[72].

Only two patients with piroxicam-induced liver toxicity were admitted to our liver unit during the last three decades (unpublished data). The first patient was a 44-year-old woman with clinical and histological evidence of submassive necrosis. The patient developed ascites and liver failure after 28 d of drug intake and uneventfully recovered without liver transplantation in 95 d from clinical onset. The other case was a 42-year-old man admitted with clinical and biochemical manifestations of severe cholestasis after piroxicam treatment as the single medication for 58 d. Liver histology showed a typical hepato-canalicular cholestasis associated with ductopenia (Figure 1). This latter case progressively evolved to prolonged cholestasis with asthenia, anorexia and intolerable itching. He underwent corticosteroids therapy for 30 d. We were able to document a complete biochemical recovery 120 d from the clinical onset.

Other oxicam derivatives were also occasionally implicated in cases of acute cholestatic hepatitis included isoxicam and droxicam[73].

The mechanism of oxicams-induced hepatotoxicity appears to be idiosyncratic and dose independent. Due to the absence of immunoallergic features in most of the reported cases, it is very difficult to support an immune-mediated mechanism of liver injury[74].

NIMESULIDE

Nimesulide has analgesic, anti-inflammatory and anti-

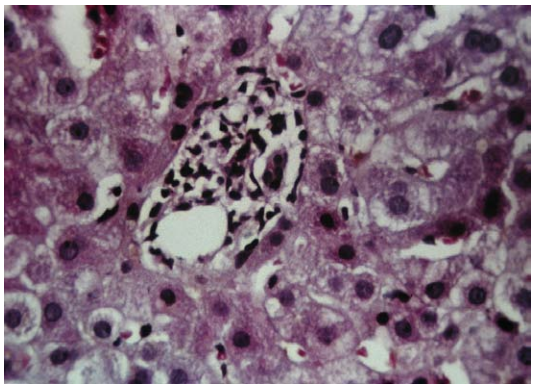

Figure 1 Piroxicam induced hepato-canalicular cholestasis associated with ductopenia (notice absence of bile duct in the portal tract).

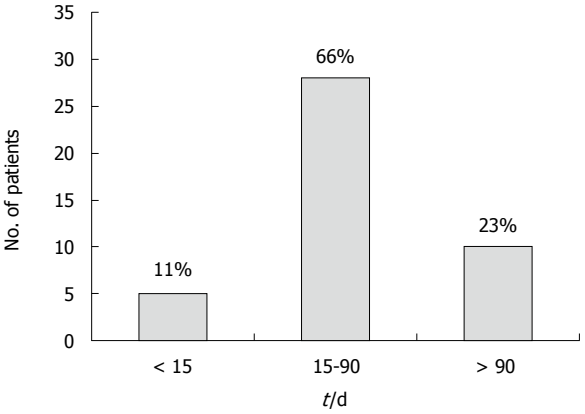

Figure 2 Latency (time from nimesulide intake to clinical onset) in 43 patients.

pyretic activity due to potent inhibitory effects on the COX-2 enzymes. Nimesulide bears a good gastro-intestinal tolerance. The mechanism of action has been attributed to a unique chemical structure of the sulphonanilides class of NSAIDs[75].

Our group in Argentina reported the first observation linking nimesulide with liver toxicity in 1997[76]. Since then, a steady flow of reports confirmed severe forms of hepatotoxicity, to the point that national health authorities of several countries withdrew nimesulide from the market[77-88]. Despite this, nimesulide commercialization is still maintained in several European countries, although the EMEA reports recommend a length of therapy restricted to 15 d and maximal dosage of 100 mg/d[13]. Controversy regarding nimesulide persists due to the fact that clinical series reports and epidemiological trials continue to involve nimesulide in severe liver damage[19,89,90]. On the other hand, health institutions conclude that nimesulide-induced-liver injury is statistically comparable to that of the remainder of the NSAIDs[91,92].

In our institution, 5 out of 30 cases (17%) had severe liver injury[93]. In 2009 our series included 43 well documented cases of nimesulide-induced liver damage associated to a wide clinical and histological spectrum of hepatotoxicity[94]. To our knowledge this constitutes the largest series of nimesulide hepatotoxicity ever reported. The

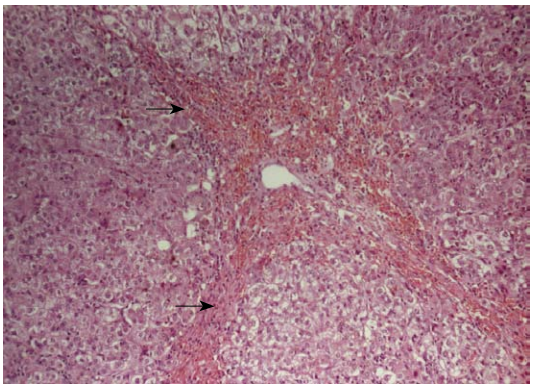

Figure 3 Acute hepatitis induced by nimesulide (hepatocellular collapsed areas are shown with arrows).

main clinical symptoms at presentation are jaundice (70%), malaise (65%) and pruritus (50%). Interestingly, two thirds of patients start liver toxicity 15 to 90 d after drug intake. Relevant to drug safety, in only 11% latency was shorter than 15 d (Figure 2). On the other hand, normalization exceeded 90 d in 27% of cases[94]. In cholestatic liver injury, normalization of alkaline phosphatase serum level usually takes more time than transaminases (i.e. more than 1 year)[95].

Nine patients in our series developed severe liver disease and FHF was observed in 6 cases. In agreement with the recent publication by Walker *et al*[96], this subpopulation was composed predominantly of females older than 50 years. Two patients died before liver transplant due to multiorgan failure, while a 9-year-old girl successfully underwent orthotopic liver transplantation.

We observed a wide range of variations of ALT/aspartate transaminase level in concordance with Bjarnason who analyzed 33 case reports documenting an elevation of ALT of at least 2-fold in 100%, and a 5-fold elevation in 89% of patients[97].

Nimesulide hepatotoxicity shows a wide spectrum of liver damage including acute hepatitis, cholestasis, mixed forms, massive and submassive hepatic necrosis. We found hepatocellular necrosis (Figure 3) in 64%, cholestatic hepatitis in 27% and pure cholestasis in 9 %[94].

The mechanism of nimesulide induced hepatotoxicity remains unknown. It has been suggested that it could be due to the formation of a reactive metabolite. On the other hand, individual genetic variations in drug metabolism have also been proposed.

Acknowledging the true impact of nimesulide on the liver is not an easy task. Despite the proliferation of reports describing nimesulide-induced severe liver injury (mainly Argentina, Ireland and Finland), the epidemiological studies have almost unanimously concluded that severe hepatotoxicity is of low incidence determining a positive risk-benefit-ratio. Inquiring about nimesulide intake should be incorporated into standard anamnesis of liver disease, especially when acute liver damage is being investigated.

Addendum

Other than the previously analyzed drugs, indomethacin,

naproxen, meloxican, tenoxican and etodoloc have also been associated with various hepatic reactions[73].

CONCLUSION

Aspirin was the first discovered NSAID. Dose dependent liver injury is accepted as the prevalent mechanism. Liver toxicity rate is very low currently since aspirin has been replaced by paracetamol and ibuprofen in pediatric patients and in various rheumatic diseases.

Diclofenac is probably the most used NSAID in rheumatology. Severe liver reactions and diclofenac hospitalization rate are uncommon. An increase in ALT levels of 3-10 × ULN is observed in 3% of cases.

Sulindac induced hepatotoxicity was documented more than fifty years ago. Liver damage occurrence was reported to be 5-10 times higher than that of other NSAIDs. A hypersensivity mechanism of liver injury was the most prevalent liver reaction.

Ibuprofen has the highest liver safety profile among NSAIDs and showed no severe liver injury in larger studies. Along with paracetamol and aspirin, it is considered one of the most common over the counter NSAIDs sold in the world.

Coxibs have currently replaced several NSAIDs due to safer GI profile. However, the high rate of cardiovascular events associated to rofecoxib is the main drawback related to drug marketing. Despite liver damage being a rare clinical situation, lumiracoxib has been discontinued in several countries due to severe hepatotoxicity.

Oxicams are associated with a well-documented hepatic safety profile. Uncommonly, piroxicam may cause severe hepatocellular damage. The clinical and histological pattern may be mixed or associated to clinical and biochemical prolonged cholestasis with or without ductopenia. Isoxicam and droxicam were only linked to liver toxicity in sporadic reports. The mechanism of liver damage appears to be an idiosyncratic one.

Nimesulide was removed from the market in several countries due to severe liver damage described in clinical series, but various epidemiological surveys do not document these findings. EMEA recommends that nimesulide should only be used for short periods at daily doses not higher than 200 mg/d in adults.

In summary, neither documenting the possibility of the causative role of a drug when confronting liver damage in an individual patient nor determining the true incidence of NSAIDs induced hepatotoxicity in the general population, are easy tasks. Rigorous data collecting, caution and clinical commitment are required when judging potential hepatotoxicity. The clinician always needs to critically evaluate the possibility whether other factors may play a role in the actual findings.

Despite the shortage of well-designed epidemiological studies, there is evidence showing that most of the NSAIDs are safe drugs with low risk of liver injury (mostly ranging from 0.29-3.1/100 000 exposed individuals when recent based-population studies were analyzed).

Both sulindac and nimesulide have been linked to a higher frequency of liver damage. NSAIDs induced liver injury which might potentially lead to a fatal outcome or need liver transplantation. As in other forms of DILI, jaundice entails poor prognosis with 25% of jaundiced patients developing severe liver disease. Drugs with an increased risk of liver damage should undergo close LFTs monitoring in order to prompt drug withdrawal to avoid severe hepatotoxicity.

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EDITORIAL

Controversy of hand-assisted laparoscopic colorectal surgery

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Abstract

Laparoscopically assisted colorectal procedures are time-consuming and technically demanding and hence have a long steep learning curve. In the technical demand, surgeons need to handle a long mobile organ, the colon, and have to operate on multiple abdominal quadrants, most of the time with the need to secure multiple mesenteric vessels. Therefore, a new surgical innovation called hand-assisted laparoscopic surgery (HALS) was introduced in the mid 1990s as a useful alternative to totally laparoscopic procedures. This hybrid operation allows the surgeon to introduce the non-dominant hand into the abdominal cavity through a special hand port while maintaining the pneumoperitoneum. A hand in the abdomen can restore the tactile sensation which is usually lacking in laparoscopic procedures. It also improves the eye-to-hand coordination, allows the hand to be used for blunt dissection or retraction and also permits rapid control of unexpected bleeding. All of those factors can contribute tremendously to reducing the operative time. Moreover, this procedure is also considered as a hybrid procedure that combines the advantages of both minimally invasive and conventional open surgery. Nevertheless, the exact role of HALS in colorectal surgery has not been well defined during the advanced totally laparoscopic procedures. This article reviews the current status of hand-assisted laparoscopic colorectal surgery as a minimally invasive procedure in the era of laparoscopic surgery.

Key words: Colorectal surgery; Laparoscopic assisted colorectal surgery; Hand assisted laparoscopic colorectal surgery

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INTRODUCTION

Since its introduction in 1991, the number of performed laparoscopic colorectal surgery has remained a minority[1-3]. This was attributed to the fact that such procedures are time-consuming, technically demanding and have a long steep learning curve[4]. In the technical demand, surgeons need to handle a long mobile organ, the colon, and has to operate on multiple abdominal quadrants most of the time with the need to secure multiple mesenteric vessels. However, the most important deterring reason was the fear that laparoscopic colectomy was considered oncologically unsound in the management of colorectal cancers[5]. Therefore, a new surgical innovation called hand-assisted laparoscopic surgery (HALS) was introduced in the mid 1990s as a useful alternative to totally laparoscopic procedures[6-8]. This hybrid operation allows the surgeon to introduce the non-dominant hand into the abdominal cavity through a special hand port while maintaining the

pneumoperitoneum[6,7]. This innovative technique met with fierce resistance and its validity was questioned. Nevertheless, there has been an upsurge in the performance of both laparoscopic and hand-assisted laparoscopic colectomy (HALC) over the past 5 years. The reasons are the introduction of more versatile laparoscopic instruments, the introduction of new vessel-sealing devices like the harmonic scalpel and Ligasure, and various laparoscopic endo-staplers. This has enabled surgeons to perform laparoscopic colorectal procedures without the need for a single intracorporeal knot or suture. Another important factor that has contributed tremendously to this upsurge is the emergence of a level I evidence confirming that the laparoscopic colorectal technique is as oncologically sound as the open procedure[9,10]. The European Cooperation in Science and Technology (COST) multi-institutional study suggested that the laparoscopic approach is an acceptable alternative to open surgery for colon cancer with a similar rate of recurrent cancer after laparoscopically assisted colectomy (LAC) and open colectomy[9]. However, the questions remain: is HALC an alternative to LAC? Is it second best? Or are they complementary to each other? This article reviews the current literature in an attempt to demonstrate the status of hand-assisted laparoscopic colorectal surgery as a minimally invasive procedure in the era of laparoscopic surgery.

ARGUMENT FOR HALC

The proponents of HALC claim that a hand in the abdomen will restore the tactile sensation which is usually lacking in laparoscopic procedures. It also improves the eye-to-hand coordination, allows the hand to be used for blunt dissection or retraction and also permits rapid control of unexpected bleeding[6-8,11,12]. All of those factors can contribute tremendously in reducing the operative time. Moreover, HALC is also considered as a hybrid procedure that combines the advantages of both minimally invasive and conventional open surgeries. It is also strongly argued that if an incision is needed to extract the resected specimen at the end of the laparoscopic procedure, then such an incision may be inflicted earlier in the procedure and be utilized as a hand port. However, this new innovation was not quickly embraced and has been fiercely rejected by the surgical community.

ARGUMENT AGAINST HALC

The opponents argued that introducing a hand in the abdomen during any laparoscopic procedure violates the fundamental principles of minimally invasive surgery (MIS) and makes maintenance of pneumoperitoneum difficult. Furthermore, if this new surgical innovation is adopted, one may witness the birth of a new generation of surgeons who is reluctant to learn totally laparoscopic techniques; a generation who will be offering their patients a "second" best procedure[13]. Also, this new innovation is more aggressive and traumatic as the incision for the hand insertion and specimen extraction is inflicted earlier in the operation with persistent and continuous stretch and compression on the wound. It is also speculated that the increased handling and mobilization of the bowel will result in the development of postoperative ileus and intra-abdominal adhesions. Moreover, there is uncertainty about the long-term results such as development of adhesive small bowel obstruction and ventral hernias, *etc*. Other arguments focused on the cost incurred by the use of the hand port, the comparatively larger size of the hand port and the extraction incision, obstruction of the operative view by the inserted hand and the ergonomics of this technique as up to 20% of surgeons reported forearm fatigue and wrist pain at the end of the procedure[14]. Hence, it is difficult to convince experienced laparoscopic surgeons to introduce a hand in the abdomen in order to speed up the procedure and it remains questionable whether there is actually a need for them to do so[13].

INDICATIONS AND CONTRAINDICATIONS OF HALC

HALC can be offered to all patients who are undergoing any form of colorectal resection for benign as well as malignant conditions. The procedure is best suited for the obese especially those with body mass index (BMI) of 40 or more, as the conversion rate is high if the procedure is conducted laparoscopically[13]. HALC is also indicated in cases where the pathology is bulky and whenever the laparoscopic surgeon is contemplating conversion of the laparoscopic procedure to an open technique due to unexpected difficulties during the procedure[13]. Similarly, it can be utilized whenever the surgeon encounters difficulty or wants to speed up the operation pace in areas where there is laparoscopic technical difficulties such as taking down the splenic or hepatic flexures. It can also be considered in cases of total colectomy when an hour saving in the operating time can be gained[13]. However, there is a doubtful advantage of HALC in low rectal surgery over the laparoscopic technique.

Therefore, generally speaking, HALC should be avoided in patients with low BMI, thin patients with a small abdomen, and in pediatric patients. It is also contraindicated when the pathology is non-bulky and the surgeon's hand is huge.

HAND PORTS

Hand ports facilitate the hand insertion; act as specimen retrieval site and also as a wound protector. They further serve as portals for construction of extracorporeal anastomoses and can also serve as laparoscopic trocar sites. The latter permits selective use of HAL and laparoscopically assisted (LA) techniques at various times during the same operation. The first generation of hand ports was cumbersome, and allows loss of pneumoperitoneum due to gas leak. This gave HALS a bad reputation in its early days. However, newer hand ports devices have better sealing mechanisms and are more user friendly abolishing

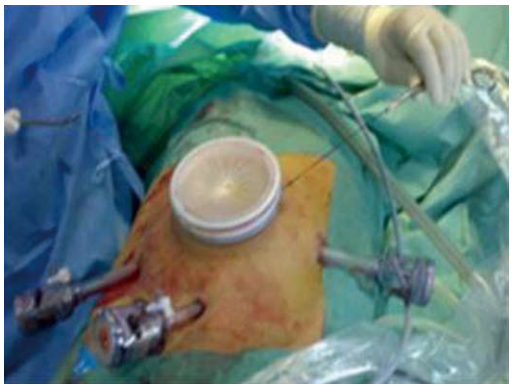

Figure 1 An operative picture showing the placement of the hand port and other trocars.

the initial criticism of the old hand ports. Currently, there are varieties of hand ports, but the most commonly used are LapDisc (Johnson and Johnson Endosurgery, USA) (Figure 1) and Gelport (Covidien, Autosuture, USA)[14,15].

Some new special finger mounted surgical instruments that may help some delicate steps of surgical procedures such as intracorporeal dissection and cutting, have been designed for HALS. However, there have been few reports about their efficiency and usefulness in clinical practice.

HALS *VS* OPEN COLECTOMY

There is no doubt that HALC is far superior to open colectomy (OC) as it preserves the advantages of MIS. There are three randomized controlled trials (RCT) that compared HALC and OC surgery[16-18]. The first RCT that compared two well matched groups: 41 patients with HALC *vs* 40 with OC undergoing elective management of right-sided colonic cancer[16] found that HALC took significantly longer time to perform (110 min *vs* 97.5 min, $P = 0.003$), but resulted in significantly less blood loss (35 mL *vs* 50 mL, $P = 0.005$) and was associated with significantly less pain, less parenteral and enteral analgesia[16]. Moreover, patients receiving HALC recovered faster, and had a shorter length of hospitalization (7 d *vs* 9 d, $P = 0.004$). The 5-year survival rate was similar between the two groups (83% *vs* 74%, $P = 0.90$)[16].

The second RCT evaluated the postoperative recovery after HAL ($n = 30$) *vs* open ($n = 30$) restorative proctocolectomy with ileal pouch anal anastomosis for ulcerative colitis and familial adenomatous polyposis[17]. The operating time was longer in the HAL group compared with the open group (210 and 133 min, respectively; $P < 0.001$). However, there were no significant differences in either narcotics requirement or morbidity or postoperative hospital stay (20% *vs* 17%, and 10 d *vs* 11 d, respectively). Moreover, there was no difference between the two procedures in quality of life (QOL) assessment score in the 3 mo after surgery. However, the HAL procedure was more costly than the open procedure[17].

In the third RCT, Kang *et al*[18] randomized 60 patients into two well-matched groups: HALC ($n = 30$) and OC ($n = 30$) for the management of benign or malignant colorectal diseases. The patients undergoing HALC had a significantly shorter hospital stay and incision length, faster recovery of gastrointestinal function, less analgesic use and blood loss, and lower pain scores on the 1st, 3rd and 14th postoperative days. Moreover, there were no significant differences in operating time, complications, or time to recover to normal[18].

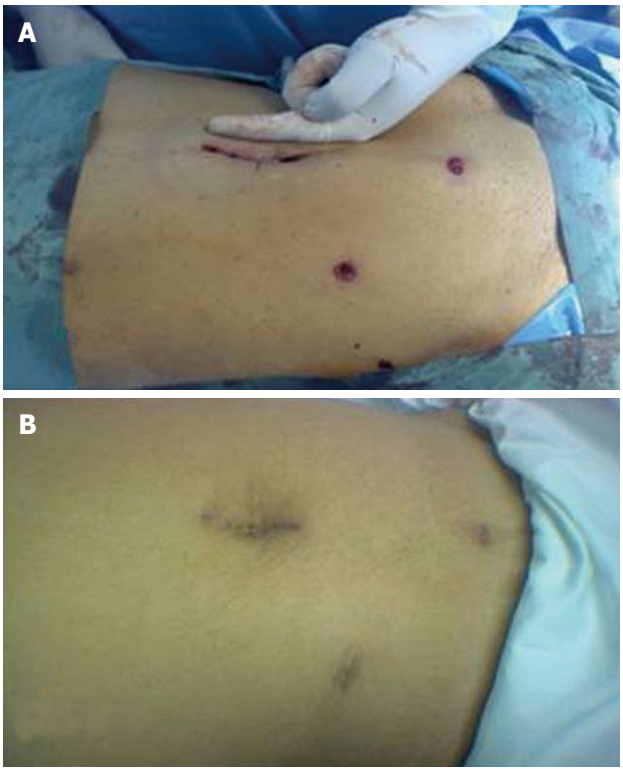

Figure 2 Cosmetic results after hand-assisted laparoscopic colectomy. A: It shows the hand port site closure and the trocar sites at the conclusion of hand-assisted laparoscopic sigmoid colectomy; B: It shows the operative wounds 2 mo after the procedure. Note the shrinkage of the port site scar and the acceptable cosmesis.

A fourth comparative study that aimed to compare the QOL, functional outcome, body image, and cosmesis after hand-assisted laparoscopic (HALRPC) *vs* open restorative proctocolectomy (ORPC) in 53 patients who completed the QOL and functional outcome questionnaires[19] found no differences in the functional outcome, morbidity, or QOL between the two groups. However, at a median of 2.7 years after surgery, the body image and cosmoses scores of female patients were significantly higher in the HALRPC group[19].

In summary of the above RCTs (Table 1), HALC takes longer time, but is associated with less blood loss, less pain, faster postoperative recovery with a shorter length of hospital stay and incision than OC. Furthermore, there is no difference in the complication occurrence, and HALC is associated with higher body image and cosmesis scores in female patients (Figure 2A and B), but is more costly than OC.

Table 1 Published studies comparing hand-assisted laparoscopic colorectal surgery with open colorectal surgery

Study, yr	Patients (HALC *vs* open)	Indication	Procedure	OT (min)	Blood loss (mL)	Analgesia narcotics or POD	Diet (POD)	Complications	LOS (d)
Maartense *et al*[17], 2004	30 *vs* 30	UC and FAP	RPIPAA	214 *vs* 133	262 *vs* 300	30 mg *vs* 31 mg	6 *vs* 7	20% *vs* 17%	10 *vs* 11
Chung *et al*[16], 2007	41 *vs* 40	Cancer	R. colectomy	110 *vs* 97	35 *vs* 50	19 mg *vs* 54 mg	3 *vs* 3	9.7% *vs* 22.5%	7 *vs* 9
Kang *et al*[18], 2004	30 *vs* 30	Benign and malignant CR diseases	Colectomies (R, L and total), AP	169 *vs* 172	193 *vs* 84	2.6 d *vs* 3.3 d	3.7 *vs* 4.4	13% *vs* 30%	8 *vs* 10

UC: Ulcerative colitis; FAP: Familial adenomatous polyposis; RPIPAA: Restorative proctocolectomy and ileal pouch-anal anastomosis; OT: Operative time; POD: Postoperative day; HALC: Hand-assisted laparoscopic colectomy; R: Right; L: Left; AP: Anterior resection; CR: Colorectal.

HALC *VS* LAC

A review of the literature yielded 8 important studies which compare HALC and LAC; 4 randomized trials[14,20-22], 2 prospective non-randomized[23,24] and 2 large retrospective studies[10,25]. The HALS study was conducted by 10 surgeons from Europe and America and included only 40 patients who were randomized into HALC (18 patients) and LAC (22 patients)[14]. This study, though small in size,found no significant difference between the two groups in term of operating time (142 min *vs* 151 min), length of incision (7.4 cm *vs* 7.0 cm), rate of major complications and length of hospital stay (7 d *vs* 6 d). However, there were fewer conversions in the HALC group (14% *vs* 22%). The HALS study group concluded that HALC retains the benefits of MIS[14]. The second study by Targarona *et al*[20] included a larger number of patients: 54 patients randomized equally into HALC and LAC groups. The operating time and clinical outcome were similar. However, the conversion rate was much higher in the LAPC group (23% *vs* 7%). Of interest, 4 of 6 conversions in the laparoscopic group were completed with the hand-assisted technique. There is another interesting finding in this study that the inflammatory (tissue injury) markers such as interleukin-6 and C-reactive proteins were increased in the hand-assisted group. This may lead us to believe that HALC is a more aggressive procedure than LAC, but preserved the features of MIS. This has also opened the door for using HALC as a halfway house procedure and adjunct to LAC when difficulties are encountered and when conversion to the open procedure is contemplated during laparoscopic colectomy[13].

The Minimally Invasive Therapy and Technology (MITT) group study[21] consisted of a multi-centre (5 hospital, 11 surgeons), unblinded RCT which compared HALC and LAC for segmental (SC) and total colectomy/proctocolectomy (TC). The HALC group (47 patients: 33 SC and 14 TC) and LAC group (48 patients: 33 SC and 15 TC) were both matched for age, sex, diagnosis, BMI and previous surgery. There was no significant difference in the complication rates (19% *vs* 21%) and long-term clinical outcome, but the extraction incision was bigger (8.2 cm *vs* 6.1 cm) and the conversion rate was lower (2% *vs* 12.5%) in the HALC group. Moreover, there were no apparent differences in the time for bowel function recovery, tolerance of diet, length of hospital stay, postoperative pain scores, or narcotic use between the two groups. Another interesting finding in this study is that the operating time can be reduced by more than 30 and 60 min in SC and TC, respectively if the procedure is conducted by HALC instead of LAC[21]. The last RCT compared 35 HALRPC and 30 LARPC[22]. There were neither conversions nor intraoperative complications, and the median operating time was longer in LAPRPC group (298 min *vs* 214 min, $P < 0.001$). Morbidity and reoperation rates were comparable (29% *vs* 20% and 17% *vs* 10%, respectively). The median hospital stay was 9 d in the laparoscopic group compared with 10 d in the HAL group. Moreover, there were no differences in QOL and the total costs[22].

A prospective non-randomized case control study on ultra-low anterior resection was reported by Tjandra *et al*[23] with an equal number of patients (32 HALC and 31 LAC). There were no conversions in both groups with similar oncological harvest in term of tumor clearance and number of lymph nodes retrieved. The length of hospital stay was the same (5.9 d *vs* 5.8 d). But, the operating time was significantly shorter in the HALC group (170 min *vs* 188 min). The duration needed for postoperative narcotics was significantly longer (3.0 d *vs* 1.5 d) and the bowel function recovery and flatus passage were delayed (3.4 d *vs* 1.9 d) in the HALC group. This study has confirmed some difference in recovery in favor of the laparoscopic group. This difference is, however, of doubtful clinical significance as the length of hospital stay is unaffected[23].

Also a prospective comparative study analyzed 258 well-matched patients undergoing HALS ($n = 109$) or LAC ($n = 149$)[24]. A significantly greater proportion of HALS patients underwent complex procedures and extensive resections. However, there were no differences in the conversion rates (15% *vs* 11%, $P = 0.44$), intraoperative complications (4% *vs* 1%, $P = 0.17$), the 30-d morbidity (18% *vs* 11%, $P = 0.12$) and surgical reinterventions (2% *vs* 1%, $P = 0.58$). There was no difference in the recovery judged by days to pass flatus (mean 3 d *vs* 3 d), however HALS took a longer operating time (276 min *vs* 211 min, $P < 0.0001$) and resulted in 1 d longer stay in hospital (6 d *vs* 5 d, $P = 0.0009$). It was concluded that HALS facilitates the expansion of a minimally invasive colectomy practice to include more challenging procedures while maintaining the short-term benefits of LAC[24].

A large retrospective single institution study from the Lahey clinic[25] comparing HAL sigmoid colectomy ($n = 66$) with LA sigmoid colectomy ($n = 85$) revealed no

Table 2 Published studies comparing hand-assisted laparoscopic colorectal surgery with laparoscopically assisted colorectal surgery

Study, yr	Patients (HALC *vs* LAC)	OT (min)	Incision length (cm)	Complications (%)	Conversion rate (%)	LOS (d)	Bowel function (d)	Comments and conclusion
HALS study[14], 2000	18 *vs* 22	142 *vs* 151	7.4 *vs* 7.0	4.5 *vs* 5.5	14 *vs* 22	7 *vs* 6	NA	HALC retains the benefits of MIS
Targarona *et al*[20], 2002	27 *vs* 27	120 *vs* 135	NA	26 *vs* 22	7 *vs* 23	POD3: 6 *vs* 6	NA	Inflammatory markers such as interleukin-6 and C-reactive proteins were raised in HALC group
MITT study[21], 2008	47 *vs* 48	163 *vs* 210	8.2 *vs* 6.1	19 *vs* 21	2 *vs* 12.5	5 *vs* 4	2.5 *vs* 3	The OT can be reduced by > 30 min and 60 min in SC and TC; respectively if conducted by HALC
Polle *et al*[22], 2008	30 *vs* 35	214 *vs* 298	NA	Major: 16.5 *vs* 20	NA	10 *vs* 9	6 *vs* 5	No significant short-term benefits for total laparoscopic compared with HALRPC with respect to morbidity, OT, QOL, costs, and LOS
Tjandra *et al*[23], 2008	32 *vs* 31	170 *vs* 188	NA	22 *vs* 25.8	0 *vs* 0	5.9 *vs* 5.8	3.4 *vs* 1.9	Some difference in recovery in favour of the laparoscopic group
Hassan *et al*[24], 2008	109 *vs* 149	276 *vs* 211	NA	18 *vs* 11	15 *vs* 11	6 *vs* 5	3 *vs* 3	HALS facilitates expansion of a MIS colectomy to include challenging procedures while maintaining short-term benefits of LAC
Chang *et al*[25], 2005	66 *vs* 85	189 *vs* 203	8.1 *vs* 6.2	21 *vs* 23	0 *vs* 13	5.2 *vs* 5	2.5 *vs* 2.8	No difference in return of bowel function, LOS or complications. Significant difference in the OT and conversion rate in favour of HALC group. The incision size was smaller in the LAC group
Ringley *et al*[10], 2007	22 *vs* 18	120 *vs* 156	7 *vs* 5.5	Similar	NA	4 *vs* 4	NA	HALC is associated with shorter OT and greater lymph node harvest, but equal I.O blood loss, pedicle length and LOS

HALC: Hand-assisted laparoscopic colectomy; LAC: Laparoscopically assisted colectomy; OT: Operative time; LOS: Length of hospital stay; NA: Not available; MIS: Minimally invasive surgery; HALRPC: Hand-assisted laparoscopic restorative proctocolectomy; QOL: Quality of life; POD3: Postoperative day 3.

significant difference in bowel function recovery (2.5 d *vs* 2.8 d), length of hospital stay (5.2 d *vs* 5.0 d) or short-term complications such as anastomotic leak, ileus and wound infection (21% *vs* 23%) between the two groups. However, there was significant difference in the operating time (189 min *vs* 203 min) and conversion rate (0% *vs* 13%) in favor of the HALC group. The incision length was, however, significantly smaller (8.1 cm *vs* 6.2 cm) in the LAC group[25].

Another retrospective review of 40 patients (22 HALC and 18 LAC) comparing conventional laparoscopic and hand-assisted oncological segmental colonic resection was reported by Ringley *et al*[10]. HALC was found to be associated with a shorter operating time (120 min *vs* 156 min, $P < 0.05$) and greater lymph node harvest (16 *vs* 8, $P < 0.05$), but equal intraoperative blood loss, pedicle length and hospital stay (4 d)[10]. LAC was completed with smaller incision length to retrieve specimen (7 cm *vs* 5.5 cm, $P < 0.05$), but this 1.5 cm difference is of doubtful clinical significance[10]. Table 2 summarizes the above studies that compare HALC and LAC and their conclusions. Based on the reviewed literature, the following results were found between HALC and LAC: (1) HALC offers the same MIS benefits as LAC; (2) HALC has a shorter operative time and lower conversion rate; (3) They both have comparable complication rate and length of stay; (4) The incision length is bigger in HALC; (5) There an increased level of inflammatory markers in HALC; (6) There is an increased need for postoperative narcotics analgesia after HALC; (7) Bowel function recovery and passage of flatus are somewhat delayed in HALC, but this is of doubtful clinical significance; and (8) HALC is more suitable for the obese patients.

IMPACT OF HALC ON SURGICAL TRAINING

It has been postulated that "It takes 6-12 mo to teach fellows how to take down the splenic flexure independently using straight laparoscopic methods whereas most fellows become proficient at the same task about HAL method after performing 10-15 cases"[13] and it was also claimed that "a surgeon who uses either straight laparoscopic or HAL methods extremely will be handicapped"[13]. It was therefore advisable that trainees embrace and master both techniques. A recent comparative study measuring the percentage of left-sided HALC or straight LAC cases completed by a trainee surgical resident found that straight laparoscopy were more likely completed by the resident without the intervention of the attending physician than HALC (LAC, 88%; HALC, 72%; $P = 0.06$)[26]. Differences in the mean operating time favoring LAC were noted (HALC, 142 min *vs* LAC, 133 min; $P = 0.04$)[26]. However, the occurrence of complications was similar in the two groups (HALC, 19% *vs* LAC, 21%), so was the rate of conversions (HALC, 5.6% *vs* LAC, 4.5%). It was concluded that trainee surgical residents may be more successful in completing LAC than in adjusting to the novel HALC approach during training. This is somewhat surprising, but it may be explained by the hindrance of

the laparoscopic view by the intervening hand during HALC, especially in the early learning curve of the trainee surgeons.

A 25-question survey organized by The American Society of Colon and Rectal Surgeons about hands-on training cadaver courses, found that a laparoscopic colon resection was performed within 1 wk of the course by 52% of participants and within 1 mo by 90%[27]. Hand-assisted technologies have lowered the threshold for performing the first LAC in 62% of participants. Most participants (77%) declared that the most important factor in the course selection was a cadaver model. It was therefore concluded that cadaver courses enabled rapid integration of laparoscopic colon resection into clinical practice and that hand-assisted technologies promoted technique acquisition[27]. The author conducted HALC workshops on live animal (sheep) which made consultant surgeons with no experience in laparoscopic colectomy more confident in using the HALS technology they obtained in the animal workshop as a bridge towards totally laparoscopic procedures in humans[15].

COST ANALYSIS

A concern has been raised regarding the higher direct cost of HALC, however, the results are inconsistent. In a RCT that evaluated postoperative recovery after HAL *vs* open restorative proctocolectomy with ileal pouch anal anastomosis for ulcerative colitis and familial adenomatous polyposis[17], the HAL procedure was found more costly than the open procedure (the median overall cost was $16.728 for HAL procedure and $13.406 for the open procedure; $P = 0.095$)[17]. In a retrospective analysis of 73 patients undergoing LAC *vs* 101 undergoing HALC[28], it was found that the operative cost and cost of consumables were higher for HALC (US$4024.2 *vs* US$3568.1, $P = 0.01$ and US$1724.7 *vs* US$1302.7, $P < 0.001$, respectively). However, the total costs were not significantly different between the two procedures (HALC US$8999.8, LC US$7910.7, $P = 0.11$). In a more recent US study that looked at direct costs for the operating room, nursing care, intensive care, anesthesia, laboratory, pharmacy, radiology, emergency services and consultation, and professional and ancillary services related to the initial hospitalization and readmissions associated with 100 HALC *vs* 100 matched LAC cases which were performed concurrently[29], there were no differences in the operating time (168 and 163 min, respectively), length of hospital stay (4 d), readmission (6% and 11%, respectively), or reoperation rates (5% and 9%). The overall morbidity was 16% and 32% for HAL and LAC, respectively ($P = 0.009$). The major morbidities, including abscess, hemorrhage and anastomotic leak, were also similar in the two procedures. However, operating room costs were increased for HALC (US$3476 *vs* US$3167); the total costs were similar (US$8521 *vs* US$8373). Therefore, it can be concluded that the total costs for HALC and LAC are similar[29] and HALC reserves the benefits of LAC at no extra cost[30].

LONG-TERM COMPLICATIONS OF HALC

As HALS requires a larger incision that that used in totally laparoscopic procedures, it has been postulated that HALS may be associated with more long-term complications such as incisional hernias and adhesive small bowel obstruction. This has been addressed by Sonoda *et al*[31] who compared HALS ($n = 270$) and LAS ($n = 270$) over a median follow-up of 27 mo (1-72 mo). Despite the larger wound in the HALS group (median 75 mm *vs* 45 mm), the incidence of incisional hernia was similar in both groups and the rate of small bowel obstruction was also comparable (4.1% *vs* 7.4%, $P = 0.11$)[31]. Moreover, the incidence of wound infections was also comparable (HALS 6.8% *vs* LAS 4.8%, $P = 0.33$). Interestingly, the converted cases had a significantly higher incidence of incisional hernia than that of the non-converted patients (25% *vs* 5%), although the rate of small bowel obstruction was the same. It was therefore concluded that HALS does not lead to more long-term complications of incisional hernia and small bowel obstruction than totally laparoscopic procedures[31].

CONCLUSION

Based on the available evidence, hand-assisted laparoscopic colorectal resection offers similar short and long-term MIS benefits to that of totally laparoscopically assisted procedures. It combines the advantages of both laparoscopic (minimally invasive) and conventional open surgery. It is safe and feasible in benign as well as malignant colorectal tumors. Furthermore, it is easy to learn, easy to teach and most useful in complex colorectal procedures. Hence, hand-assisted colorectal surgery is advocated first as a 'bridge' and later as an adjunct to laparoscopically assisted colorectal procedures. Moreover, it can be used as an alternative to laparoscopic colectomy in the complex colorectal procedures.

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TOPIC HIGHLIGHT

Yeng S Ang, Dr., MD, *Series Editor*

Biomarkers in Barrett's esophagus and esophageal adenocarcinoma: Predictors of progression and prognosis

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Abstract

Barrett's esophagus is a well-known premalignant lesion of the lower esophagus that is characterized by intestinal metaplasia of the squamous epithelium. It is clinically important due to the increased risk (0.5% per annum) of progression to esophageal adenocarcinoma (EA), which has a poor outcome unless diagnosed early. The current clinical management of Barrett's esophagus is hampered by the lack of accurate predictors of progression. In addition, when patients develop EA, the current staging modalities are limited in stratifying patients into different prognostic groups in order to guide the optimal therapy for an individual patient. Biomarkers have the potential to improve radically the clinical management of patients with Barrett's esophagus and EA but have not yet entered mainstream clinical practice. This is in contrast to other cancers like breast and prostate for which biomarkers are utilized routinely to inform clinical decisions. This review aims to highlight the most promising predictive and prognostic biomarkers in Barrett's esophagus and EA and to discuss what is required to move the field forward towards clinical application.

Key words: Barrett's esophagus; Esophageal adenocarcinoma; Esophageal dysplasia; Prognosis

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INTRODUCTION

Barrett's esophagus is defined as an esophagus in which the distal portion of the normal squamous lining has been replaced by a metaplastic columnar epithelium. In order to make a diagnosis of Barrett's esophagus, a segment of columnar metaplasia of any length must be visible endoscopically above the esophagogastric junction and be confirmed or corroborated histologically[1]. This condition usually develops in the context of longstanding, severe gastroesophageal reflux disease (GERD)[2], and is the only recognized precursor lesion for development of esophageal adenocarcinoma (EA). The incidence of EA arising from Barrett's esophagus is variable, depending on the grade of dysplasia associated with it. The risk of progression to cancer increases gradually from 0.5% per year for non-dysplastic Barrett's, to 13% in low-grade dysplasia (LGD) and 40% in high-grade dysplasia (HGD)[3,4].

In Barrett's esophagus, it is widely accepted that there are three main histological subtypes. They include epithelium that comprises mainly a gastric fundus subtype with parietal and chief cells; a junctional (cardia) subtype with mucus-secreting glands; and the distinctive metaplastic columnar epithelium with intestinal-type goblet cells[1,5]. These three histological subtypes occupy different zones in the esophagus. The intestinal-type metaplasia with goblet cells is found most proximally next to the squamous epithelium, followed by the junctional (cardia) subtype in the middle, and the gastric fundus subtype most distally. The relevance of this subgrouping of the histological subtypes of Barrett's esophagus lies in the potential to develop malignancy. The fundic subtype has a very low risk of developing EA malignant potential, whereas the metaplastic columnar epithelium with intestinal-type goblet cells and the junctional (cardia) type have a more significant risk of malignant transformation[6,7]. This concept is important as this together with the problem of defining Barrett's esophagus based on location and length of metaplastic epithelium has led to a detailed discussion in the American Gastroenterological Association Institute technical review on Barrett's esophagus. This meeting redefined Barrett's esophagus as "the condition in which any extent of metaplastic columnar epithelium that predisposes to cancer development replaces the stratified squamous epithelium that normally lines the distal esophagus"[8]. However, it is slowly becoming apparent that the risk for development of EA is not solely limited to the intestinal type and that better designed and powered studies are required to assess properly the true risk of progression in each subtype[9].

During the development of EA, the epithelium accumulates multiple molecular abnormalities and becomes increasingly dysplastic[10]. The diagnosis of dysplasia allows the progression from Barrett's esophagus to EA to be monitored by endoscopic surveillance biopsies with the aim of intervening prior to the development of invasive adenocarcinoma. Although randomized controlled evidence is lacking, EA detected *via* this strategy appears to confer a much better prognosis, as surveillance detected disease is often at an early stage prior to lymph node involvement[11,12].

There are a number of problems with this current clinical algorithm. First of all, a significant proportion of patients with Barrett's esophagus are undiagnosed[13-16], and therefore, will not benefit from any cancer prediction strategies. Second, surveillance is not proven to reduce population mortality and is based on the subjective assessment of dysplasia, which has inter and intra-observer error[17-19]. Lastly, because most patients with Barrett's esophagus are at extremely low risk of developing EA[20], the majority are having unnecessary surveillance, which is cumbersome both for the clinician and the patient, and poses a strain on the healthcare system. A recent review to assess the cost-effectiveness of surveillance of Barrett's esophagus based on a Markov model has revealed that surveillance of Barrett's esophagus for all grades of dysplasia does more harm than good when compared to no surveillance[21]. This report has suggested that surveillance does not produce more quality-adjusted life years than no surveillance, and that there is no apparent survival advantage of cancer detected by surveillance due to a high recurrence rate and increased mortality from surgical interventions. It is hoped that biomarkers assayed in readily obtainable biological samples, such as blood or endoscopic biopsies, can be identified to improve the clinical management at each stage in the disease. Screening biomarkers could enable unidentified cases of Barrett's esophagus to be diagnosed in the population (Figure 1, green arrow), whereas predictive biomarkers could be used as adjuncts or to replace the current surveillance program for the detection of dysplasia, as well as potentially being able to predict which patients are at high risk of developing cancer in the future (Figure 1, blue arrow). For patients presenting *de novo* with EA, prognostic biomarkers could be useful to determine the best therapeutic approach and prognosis (Figure 1, red arrow). In addition, biomarkers might have a role in determining response to treatment including chemopreventive agents, endoscopic treatments for patients with Barrett's, and the use of molecular targeted therapy for those with cancer.

CLINICAL BIOMARKERS

Clinical biomarkers can be defined as a characteristic that can be objectively measured or evaluated as an indicator of normal biological processes, pathological processes or a response to a therapeutic intervention[22]. Importantly, the quantification of biomarkers should aid, improve or alter clinical management. The criteria required for adoption of biomarkers into clinical use are not well defined. Therefore, the Early Detection Research Network (EDRN) has defined five stages for development of biomarkers for risk of progression[23] and similarly, McShane *et al*[24] have recently published recommendations for prognostic tumor marker development (Figure 2). Despite the recommendation of different robust algorithms for biomarker development, fewer than 12 biomarkers have been approved by the US Food and Drug Administration for monitoring response, surveillance and recurrence of cancer at the current time[25]. This is alarming as thousands of biomarkers have been declared to be useful for diagnosis, surveillance or therapeutic markers for diseases. Most of these biomarkers do not progress to clinical practice either due to problems developing accurate assays or because the biomarker lacks sufficient sensitivity and specificity in validation studies[26]. Clearly, a large concerted effort is needed to advance the field of biomarker discovery and clinical implementation.

Biomarkers in Barrett's esophagus and EA are mostly selected due to their role in carcinogenesis. It is clear that during the transition from metaplasia to carcinoma, many molecular alterations take place and they operate together to influence the pathogenesis of dysplasia and EA. Biomarkers can be identified and investigated for their clinical applicability using two different complementary

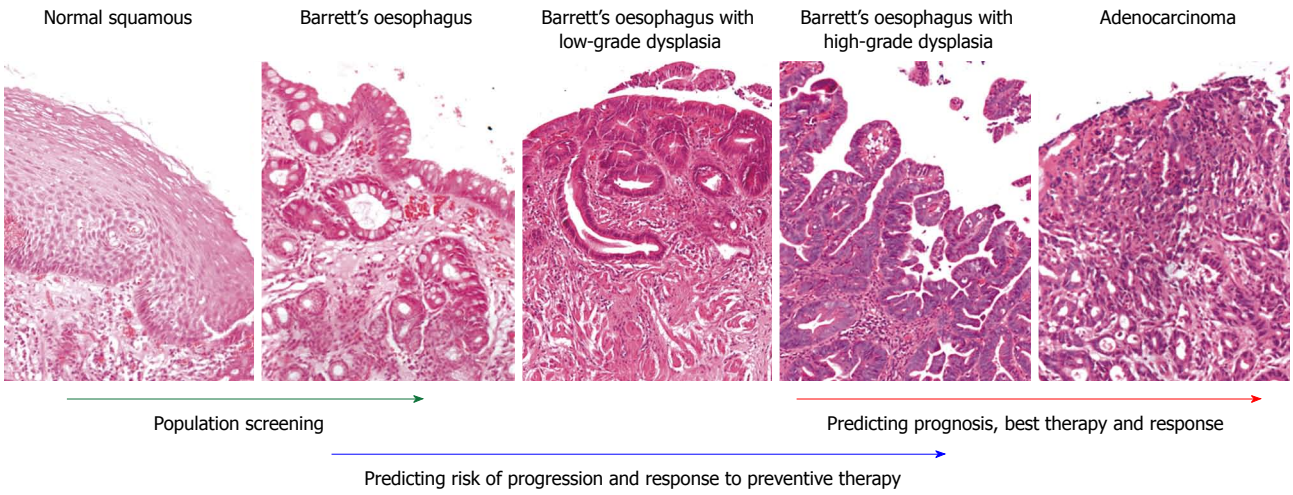

Figure 1 Transition of squamous epithelium to intestinal metaplasia, dysplasia and adenocarcinoma, with potential useful biomarkers at each stage of the disease. The left-most panel shows normal stratified squamous epithelium. The second panel shows Barrett's esophagus without dysplasia, with the presence of goblet cells. The third and fourth panels show Barrett's esophagus with low-grade dysplasia and high-grade dysplasia, whereas the last panel shows adenocarcinoma.

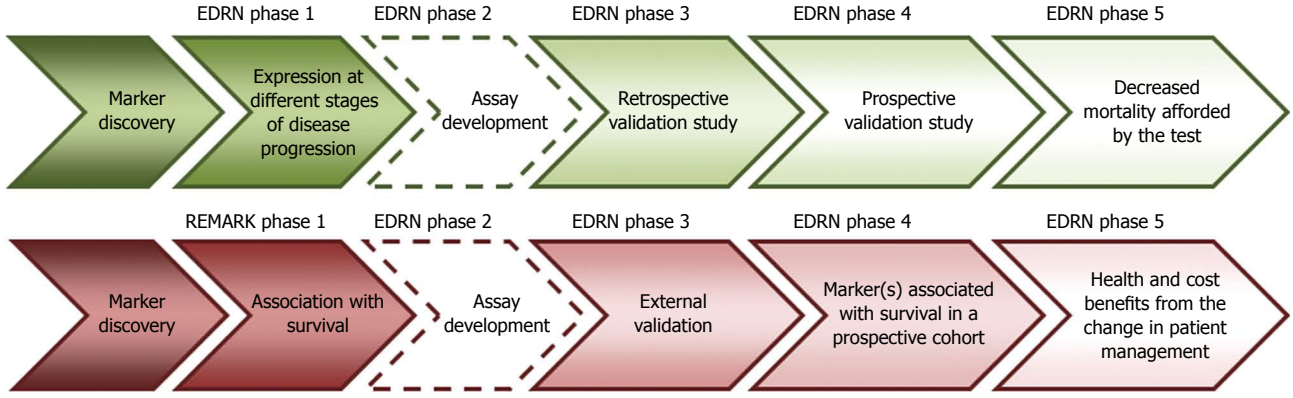

Figure 2 Phases of diagnostic and prognostic biomarker development proposed by the Early Detection Research Network and reporting recommendations for tumor marker prognostic studies before clinical implementation[23,24]. EDRN: Early Detection Research Network; REMARK: Reporting recommendations for tumor marker prognostic studies.

approaches[27]. The first approach is to identify candidate biomarkers from what is currently understood about the disease process. This is a comparatively inexpensive way to identify putative biomarkers and possibly allow for faster clinical implementation of the biomarker. The second method is to use a global screening approach without an *a priori* hypothesis. This has become possible due to the rapid expansion of "omics" technologies, including gene expression analysis, epigenetics, proteomics and single nucleotide polymorphism (SNP)-based platforms. The availability of microarray databases and other datasets on the internet also allows for the interrogation of multiple datasets to identify potential biomarkers. For example, Lao-Sirieix *et al*[28] have identified trefoil factor 3 (TFF3) as a promising biomarker to screen asymptomatic patients for Barrett's esophagus by comparing three publically available microarray databases. However, this approach requires an intensive validation process due to the potential for false discovery and can potentially be expensive and not reproducible between laboratories.

This review focuses on two main areas: (1) biomarkers predictive of progression in Barrett's esophagus, which it is hoped could transform the current surveillance program; and (2) prognostic biomarkers in EA.

PROMISING BIOMARKERS IN SURVEILLANCE OF BARRETT'S PATIENTS

Many biomarkers aimed at predicting progression in Barrett's patients have emerged over several years of research because it is appreciated that current clinical and endoscopic criteria are unable to predict which patients are likely to progress to EA. Biomarkers in Barrett's esophagus can be used for population screening and early detection of disease, confirmation of diagnosis of disease and prediction of risk of progression, which determine the prognosis of patients once adenocarcinoma develops and predict the effectiveness of therapy. Table 1 shows a summary of the biomarkers that have been most extensively investigated and their potential as clinical biomarkers. In studies evaluating the efficacy of the proposed biomarkers to determine the risk of progression from Barrett's

Table 1 Summary of the most promising biomarkers for identifying patients with Barrett's esophagus at high risk of developing esophageal adenocarcinoma

Surveillance biomarker	Highest EDRN stage	Study size (*n*)[1]	Findings	Statistical significance	Ref.
HGD	4	15	Progression to EA in 4 out of 15 patients with unifocal HGD	RR not available	[29]
		485	20 patients with HGD treated with omeprazole only developed EA	RR not available	[30]
		327	33 out of 76 patients with HGD developed EA	RR 28 (95% CI: 13-63)	[31]
		1099	12 out of 75 patients with HGD developed EA	RR 12.1 (95% CI: 5-29.4)	[32]
Aneuploidy and LOH (Reid Panel)	4	243	Panel of biomarkers (LOH of 17p and 9p and DNA abnormalities) can best predict progression to EA	RR 38.7 (95% CI: 10.8-138.5)	[33]
			LOH of 17p alone	RR 10.6 (95% CI: 5.2-21.3)	
			LOH of 9p alone	RR 2.6 (95% CI: 1.1- 6.0)	
			Aneuploidy alone	RR 8.5 (95% CI: 4.3-17.0)	
			Tetraploidy alone	RR 8.8 (95% CI: 4.3-17.7)	
p53 positivity by immunohistochemistry	3	164	Diffuse or intense TP53 staining elevated in patients who developed EA compared to controls	OR 11.7 (95% CI: 1.93-71.4)	[34]
		48	3 out of 5 patients with low grade dysplasia who progressed to high grade dysplasia had positive p53	RR not available	[35]
Mcm2	3	27	Ectopic luminal surface expression predictive of progression to HGD or EA	OR 136 (95% CI: 7.5-2464)	[36]
Cyclin A	3	48	Ectopic luminal surface expression predictive of progression to HGD or EA	OR 7.6 (95% CI: 1.6-37)	[37]
Methylation markers	3	53	Hypermethylation of *p16* (cyclin-dependent kinase inhibitor 2A), *RUNX3* (Runt-related transcription factor 3) and HPP1 (transmembrane protein with EGF-like and two follistatin-like domain 2) associated with an increased risk of progression to high grade dysplasia or EA	OR 1.74 (95% CI: 1.33-2.2), 1.80 (95% CI: 1.08-2.81) and 1.77 (95% CI: 1.06-2.81), respectively	[38]
		195	A 8 gene methylation panel in combination with age could predict half of progressors to HGD or EA who would not have been diagnosed without the use of the panel	RR not available	[39]

[1]Study size includes all patients in study and findings are extracted when relevant. Mcm2: Minichromosome maintenance protein 2; EA: Esophageal adenocarcinoma; HGD: High-grade dysplasia; LOH: Loss of heterozygosity; EDRN: Early Detection Research Network; RR: Relative risk; OR: Odds ratio; CI: Confidence interval.

esophagus to dysplasia and cancer, the odds ratio and relative risks are included whenever data were available in order to give a representation of the usefulness of the biomarkers.

DYSPLASIA

Dysplasia has been assessed as part of routine clinical practice for > 20 years. Although the assessment of dysplasia cannot be measured objectively, it is still considered a biomarker by most institutions, and is the current gold standard for determining the risk for cancer progression. The current dysplasia grading system is the Vienna classification, which divides patients into no dysplasia, LGD and HGD[40]. Due to its routine use, very few studies have been performed to document formally its predictive power. A recent meta-analysis has shown that the incidence of EA in patients undergoing surveillance for Barrett's esophagus rises in a stepwise manner using dysplasia as a biomarker. The incidence of EA was reported to be 5.98 per 1000 patient years, 16.98 per 1000 patient years and 65.8 per 1000 patient years in Barrett's patients without dysplasia, and with LGD and HGD, respectively[4]. However, histological differentiation of the different grades of dysplasia in Barrett's patients presents one of the most difficult tasks for the pathologist. In one study, 50% of Barrett's patients who were identified to have LGD by general pathologists were misdiagnosed. Forty-two percent of these misdiagnosed cases had only Barrett's esophagus without dysplasia, and 8% had HGD[41]. It is clear that histological differentiation between non-dysplastic Barrett's esophagus and LGD in particular is fraught with difficulties with poor intra- and inter-observer agreement.

HGD is known to be a surrogate marker for the high likelihood of progression to EA. Following diagnosis of HGD, endoscopic or surgical intervention is usually considered. Therefore, confirmation by two independent pathologists is a pre-requisite. As a result of the practice for intervention once HGD is detected, data on progression to EA have become much harder to obtain. Studies have shown that the risk of progression to EA ranges from 16% to 59%[31,32] and a proportion of patients in whom HGD is detected will already harbor invasive adenocarcinoma[29,32], although with intensive biopsy protocols and high definition endoscopes, this should no longer be so likely. A more ideal biomarker would be one that is less subjective and that appears earlier in the pathogenetic process, so that intervention could be considered for the highest risk patients earlier in the course of their disease. The evaluation of dysplasia is now well established and it has been suggested that other promising biomarkers are more likely to be used in conjunction with the current system than to replace the histopathological assessment of dysplasia[42].

DNA CONTENT ABNORMALITIES AND LOSS OF HETEROZYGOSITY

The use of DNA content abnormalities (aneuploidy and tetraploidy) and loss of heterozygosity (LOH) as biomarkers to predict progression of Barrett's esophagus to EA has been intensively studied by the Reid group. DNA content abnormalities are a well-known phenomenon in cancer biology. A normal cell contains 46 chromosomes, commonly referred to as 2N, and aneuploidy refers to the state in which cells have an abnormal number of chromosomes. Tetraploidy, on the other hand, specifically refers to cells that have double the number of chromosomes compared to normal cells (4N). In Barrett's esophagus, numerous studies have correlated aneuploidy and specific DNA abnormalities with the progression of Barrett's esophagus to EA[31,43-45], with Reid *et al*[31] producing the best results by combining DNA content abnormalities with LOH. Galipeau *et al*[44] have demonstrated that increased 4N (G2/tetraploid) cell populations predict progression to aneuploidy, and that the development of 4N abnormalities is interdependent with inactivation of the *p53* gene. Using flow cytometry and histology in a systematic endoscopic biopsy protocol, Reid *et al*[31] first described the use of aneuploidy and increased 4N fractions as biomarkers to identify subsets of patients with Barrett's esophagus at low and high risk of developing EA. Using a cut-off for 4N fractions of > 6% as abnormal, Reid has reported that the relative risk of cancer for these patients compared to those below this cut-off value was 7.5 (95% CI: 4-14). In addition, patients who had baseline aneuploidy had a relative risk of cancer of 5 (95% CI: 2.7-9.4) compared to patients who did not have baseline aneuploidy.

p16 and *p53* are two commonly studied tumor suppressor genes that reside on chromosome 9p and 17p, respectively. These two tumor suppressor genes can be silenced *via* LOH, mutations and DNA methylation. Silencing of the p16 allele is thought to be one of the earliest events in Barrett's esophagus, which results in clonal expansion[46]. However, a recent study by Leedham *et al*[47] has demonstrated that Barrett's esophagus can arise from multiple independent clones, which results in clonal heterogeneity. This study was performed by investigating individual crypts microdissected from esophagectomy specimens that contained adenocarcinoma and associated dysplasia, to detect clonal heterogeneity not detected by whole biopsy analysis. Overall, *p16* by itself is unlikely to be an ideal biomarker to predict progression because it appears too early in the pathogenesis, and it has been shown that there is no evidence of association between silencing of *p16* and grade of dysplasia[46]. *p53* LOH, on the other hand, provides one of the most promising biomarkers to predict progression of Barrett's esophagus, as part of the Reid panel. *p53* is a nuclear tumor suppressor protein that is responsible for the integrity of the genetic sequence. Any damage to DNA should result in increased expression of *p53*, which causes cells to arrest at the G1 phase to allow for DNA repair, and if this is not possible, then apoptosis ensues. Silencing of *p53* can occur *via* LOH or mutation of the genetic sequence, thus removing the self repair mechanism. Reid *et al*[48] have performed a prospective cohort study in 325 patients with Barrett's esophagus, and have demonstrated that LOH of chromosome 17p(p53) significantly increased the risk of progression to cancer (relative risk of 16, 95% CI: 6.2-39). In addition, Galipeau *et al*[33] have demonstrated that LOH of 17p can be combined with LOH at 9p, DNA content abnormalities and aneuploidy to form a panel of biomarkers to predict better progression of Barrett's esophagus. This panel of biomarkers provides the best predictor of progression to EA to date (relative risk of 38.7, 95% CI: 10.8-138.5). Each individual marker in the panel could in itself predict progression to EA with varying RR (Table 1), but when combined together in the Reid panel, they can most accurately predict progression to EA.

The panel of biomarkers that incorporate DNA abnormalities and LOH, which have been developed by the Reid group, are not easy to apply to the clinical setting. Efforts have therefore been made to develop alternatives. Fang *et al*[45] and Vogt *et al*[49] have tried to circumvent the problem of a high level technical expertise being required and the laboratory variability associated with flow cytometry, by using image cytometric DNA analysis in smaller studies. In these studies, they have concluded that image cytometry can provide a more sensitive marker than using HGD to identify groups of patients who are likely to progress to EA, and have highlighted that image cytometry has significant advantage over flow cytometry in terms of costs and practicality. These findings, while promising, still require validation with a much larger sample size. The development of high-fidelity DNA histograms generated by automated software to measure aneuploidy further strengthens the role of DNA abnormalities as a biomarker to predict progression in Barrett's patients[50,51]. Other interesting novel techniques to measure aneuploidy and other chromosomal aberrations have also been described in the literature. Li *et al*[52] have demonstrated that the number of SNPs was highly correlated with chromosomal abnormalities in Barrett's esophagus and EA, and have suggested that SNP-based genotyping could possibly be used to stratify the cancer risk in patients with Barrett's esophagus.

As mentioned previously, the use LOH as biomarkers is not without its own problems. The detection of LOH is complex and requires the collection of snap frozen samples, followed by extraction of DNA and an amplification step prior to polymerase chain reaction analysis[53]. This is in addition to the high costs needed to build and maintain facilities to enable the use of this panel of biomarkers in routine medical institutions. An alternative method would be to use fluorescence *in situ* hybridization (FISH) to detect LOH, but this method is limited by poor sensitivity (68.4%) when compared to genotyping[54].

Immunostaining for *p53* provides another alternative to genotyping of chromosome 17p to predict progression of Barrett's esophagus because the presence of *p53* mutations can often cause protein accumulation, which allows for detection by immunohistochemistry[34,35]. Although

the use of immunostaining of *p53* allows easy clinical implementation, its efficacy as a biomarker is limited, and positive staining was only seen in one third of patients in a nested case-control study to evaluate the efficacy of immunostaining for *p53* as a marker to predict progression[34]. This is because staining for *p53* does not always correlate with mutations. In instances in which mutations result in deletion or truncation of *p53*, it will not be detected by immunostaining.

In summary, the detection of aneuploidy and DNA content abnormalities in the Reid panel appears to be one of the most promising biomarker panels to detect the progression of Barrett's esophagus to EA. However, technical difficulties that have hindered the use of analysis of DNA content abnormalities in the Reid panel need to be addressed. SNP analysis or image cytometry are other alternative techniques used to measure aneuploidy and other chromosomal aberrations but remains to be validated in larger studies.

PROLIFERATION MARKERS

Dysplasia is typically described as being associated with abnormal cellular proliferation and differentiation[55,56]. Our laboratory and others have demonstrated abnormal surface staining of markers of proliferation [minichromosome maintenance protein (Mcm) 2, 5 and Ki67] in dysplastic Barrett's mucosa[36,55,56]. This finding has served as the basis for the use of aberrant surface expression of Mcm2, together with a brushing technique to predict progression in patients with Barrett's esophagus[36]. However, large prospective studies are needed before they can be used in routine clinical practice.

CELL CYCLE MARKERS

Members of the cyclin family such as cyclin A and D are also interesting biomarkers for Barrett's esophagus. Cyclin D is a proto-oncogene protein and overexpression in Barrett's esophagus results in inappropriate phosphorylation and inactivation of p105-Rb. Increased expression of cyclin D has been implicated in the predisposition to transform from metaplastic epithelium to cancer. and can possibly be a useful biomarker in identifying patients with Barrett's esophagus at high risk of developing EA[57,58]. Bani-Hani *et al*[58] have performed a case-control study and have shown that Barrett's patients who are positive for cyclin D detected *via* immunohistochemistry were more likely to develop EA (OR: 6.85, 95% CI: 1.57-29.91). These findings were however not replicated in a larger population-based case-control study performed by Murray *et al*[34]. In that study, only immunohistochemical detection of p53 has been shown to be a useful biomarker for malignant progression in Barrett's esophagus. Cyclin A is expressed just before the beginning of DNA synthesis and is an important check mechanism in the G1-S transition of the cell cycle. In a case-control study, surface expression of cyclin A in Barrett's esophagus samples has been shown to be correlated with the degree of dysplasia, and patients with biopsies that express cyclin A at the surface were more likely to progress to EA than those who did not (OR: 7.5, 95% CI: 1.8-30.7)[37]. Prospective studies are required to determine properly the usefulness of cyclins as predictive biomarkers.

EPIGENETIC CHANGES

Epigenetic changes (or non-DNA sequence changes) in the form of hypomethylation, hypermethylation and alteration to histone complexes have also been found to be implicated in the pathogenesis of Barrett's esophagus and EA[38,59]. Hypermethylation of promoter Cpg island is thought to be the cause of transcriptional silencing of tumor suppressor genes such as *CDKN2A* (*p16*), *APC*, *CDH1* (E-cadherin), and *ESR1* (*ER*, estrogen receptor α)[59]. Hypermethylation of these genes is usually found in a large contiguous field, which suggests possible clonal expansion of hypermethylated cells or hypermethylation of a field of metaplastic cells[59]. Further work on the methylation status of promoter regions of genes has revealed that methylation of *p16* (OR: 1.74, 95% CI: 1.33-2.20), *RUNX3* (OR: 1.80, 95% CI: 1.08-2.81) and *HPP1* (OR: 1.77, 95% CI: 1.06-2.81) in patients with non-dysplastic Barrett's esophagus and LGD were independent risk factors for progression to HGD and EA[38]. More recently, Jin *et al*[60] have demonstrated that a methylation biomarker panel that comprises eight genes could accurately determine the risk of progression in patients with Barrett's esophagus in a retrospective, multicenter validation study. In that study, promoter methylation levels of eight genes were quantified by methylation-specific PCR in patients who did not progress (n = 145) compared to those who did progress (n = 50) to HGD or EA. Receiver operating characteristics curves were constructed to evaluate the usefulness of the eight-gene methylation panel and the authors have concluded that, with specificity set at 0.9, the eight-gene methylation panel in combination with age predicted half the progressors who would not have been diagnosed without using these biomarkers. Similarly, a recent study by Wang *et al*[61] has shown that hypermethylation of p16 and APC was a good predictor of progression to HGD or EA [OR: 14.97, 95% CI (1.73, inf)]. The fact that methylation changes in DNA occur early in the progression from Barrett's esophagus to dysplasia suggest that they could potentially be used as biomarkers to predict which groups of patients are likely to progress to dysplasia and EA[59,62]. However, the main problem of the utility of hypermethylation as biomarkers lies in the fact that techniques that have been applied for detection of epigenetic changes require enzyme digestion, affinity enrichment or bisulfite treatment before probe hybridization or sequencing can be done to detect methylation in samples. These arrays of techniques are far too technically demanding and time consuming for routine utilization in the clinic[63-72].

PROGNOSTIC BIOMARKERS IN EA

The overall 5-year survival for EA remains < 14%[73].

The current staging of EA is the internationally recognized TNM system[74], which is based exclusively on the anatomical extent of the disease. This is assessed using a combination of tumor depth (T), number of lymph nodes involved (N), and presence or absence of metastasis (M). The TNM system remains useful for staging of esophageal tumors because patients with more advanced stage disease clearly do worse than those in the early stage of the disease. For patients deemed to have potentially curative disease (T3N1 or less), surgical treatment with or without chemotherapy provides the only chance of cure, but it is highly invasive and has a high morbidity rate. Biomarkers that can accurately predict the prognosis of patients with this disease could aid in the selection of patients most likely to benefit from surgery. In addition, it is also hoped that biomarkers can identify different subgroups of tumors that will benefit from specific treatment, including molecularly targeted treatments.

Prognostic biomarkers in patient with EA have commonly been studied to determine the association with the following outcome and tumor characteristics: (1) survival; (2) lymphovascular invasion and metastasis; and (3) response to chemotherapy and radiotherapy.

Traditional candidate approaches for analyzing gene and protein expression in cancer have identified a large number of biomarkers that have important prognostic value. These biomarkers can be considered in terms of the six classical hallmarks described by Hanahan *et al*[75], with inflammation added as the seventh hallmark recently. They include: (1) self sufficiency in growth signals; (2) insensitivity to growth inhibitory (antigrowth) signals; (3) evasion of programmed cell death (apoptosis); (4) limitless replicative potential; (5) sustained angiogenesis; (6) invasion and metastasis; and (7) cancer-related inflammation.

Table 2 gives an overview of the biomarkers in each category and their association with survival or surrogate measures of prognosis. This list is not exhaustive but it highlights the important biomarkers that have been investigated and reported to be prognostic. A recent review by Lagarde *et al*[76] has described in greater detail many of these biomarkers and their molecular basis. It is well known that many of these molecular alterations occur in tandem during the progression of Barrett's esophagus to EA and are present to varying degrees. These biomarkers have been shown to be associated with survival or tumor characteristics, but subsequent replication of findings, as required for the EDRN validation of biomarkers, is often lacking. It is highly unlikely that any of these markers by itself can predict survival accurately because several molecular alterations can operate together to influence the pathogenesis of EA. Again, generating panels of biomarkers to create a molecular signature in EA could be useful in determining the prognosis of patients with EA.

MOLECULAR SIGNATURE OF EA

Several studies have used microarray technologies to generate molecular signatures that correlate with overall survival, lymph node involvement or response to chemotherapy. The advantage of using these methods is that they allow the hypothesis-free interrogation of many targets simultaneously. Table 3 gives a summary of the molecular signatures discovered by microarray technology, including the methodology used. However, despite the number of studies, none of these molecular signatures or techniques to stratify patients with EA has yet reached clinical utility. This is in contrast to other cancers for which prognostic signatures are starting to be used in the clinical setting[101-103]. In EA, molecular signatures have usually been generated from underpowered cohorts and many studies have combined molecular profiling of both EA and squamous cell carcinoma of the esophagus in the same study. It is known that the molecular profile of squamous cell carcinoma and EA is different[104,105], and for accurate prognosis, studies should differentiate between these two types of tumors. An additional problem, which is not dissimilar to biomarkers discovered for the transition of Barrett's esophagus to EA, is that the technique used might not be applicable to routine laboratories and will therefore be expensive. It is therefore important that researchers also consider how best to apply molecular biomarkers to the clinic, and they should consider validation using methods such as immunohistochemistry. Whichever method is used, data reproducibility and validation in independent samples are perhaps the most important factors to determine whether molecular signatures are adopted for clinical application. This problem is becoming increasingly recognized, and many reviews have reiterated the need for validation of molecular signatures and the development of assays that have general clinical applicability[106-112].

CONCLUSION

The pathogenesis from Barrett's esophagus to EA is highly complex. Multiple molecular alterations occur during this process, which leads to a heterogenous tumor by the time that EA develops. Biomarkers can complement the current clinical management of Barrett's esophagus and its transition to EA in three main ways. They can be used to: identify patients not previously diagnosed with Barrett's esophagus *via* population screening; improve the surveillance of patients with Barrett's esophagus; and identify prognostic groups and best therapy once EA develops.

There has already been a tremendous amount of research done to create an ideal biomarker or panel of biomarkers to predict accurately progression of Barrett's esophagus to dysplasia or EA. This is in conjunction with large amounts of resources and money spent in laboratories and in clinical trials as the research is being conducted. Although no biomarkers have been able to replace the current gold standard of dysplasia as a biomarker in routine clinical practice, it is reassuring to know that certain biomarkers hold great promise to transit from the bench to the bedside. It is becoming increasingly clear that one biomarker by itself is highly unlikely to predict progression with high sensitivity and

Table 2 Summary of the biomarkers and the prognostic impact in esophageal adenocarcinoma

Category of cell alteration	Biomarker	Sample size (*n*)	Endpoint	Findings	Statistical significance	Ref.
Self sufficiency in growth signals	Cyclin D	124	Survival	2 of 3 genotypes confers a poorer overall survival	$P = 0.0003$	[77]
	EGFR	103	Survival	Expression showed a trend towards a correlation with poorer overall survival	$P = 0.07$	[78]
		75	Survival	Decreased expression correlated with poorer survival on univariate analysis only	$P = 0.034$	[79]
	Ki-67	59	Survival	Low levels (< 10%) of staining correlated with poorer survival	HR: 3.9, $P = 0.02$	[80]
	Her2/neu	63	Survival	Amplification detected by FISH correlated with poorer survival	$P = 0.03$	[81,82]
	TGF-α	61	Survival	Low levels significantly correlated with cancer specific death	$P = 0.03$	[83]
		87	Tumor progression, lymph node metastasis	High levels significantly correlated with:		
				Tumor progression	$P = 0.025$	[84]
				Lymph node metastasis	$P < 0.05$	
Insensitivity to growth inhibitory (antigrowth) signals	TGF-β1	123	Survival	Overexpression correlated with poorer survival on univariate analysis only	$P = 0.0255$	[85]
		57	Survival	High plasma levels correlated with poorer overall survival	$P = 0.0317$	[86]
	APC	52	Survival	High plasma levels of methylation of APC associated with poorer survival	$P = 0.016$	[87]
	P21	30	Survival	Alteration in expression after chemotherapy correlated with better survival	$P = 0.011$	[88,89]
Evasion of programmed cell death (apoptosis)	P53	30	Survival	Alteration in expression after chemotherapy correlated with better survival	$P = 0.011$	[88]
	Bcl-2	35	Survival	Expression correlated with poorer survival	$P = 0.03$	[90]
	COX-2	100	T-stage, N-stage, tumor recurrence and survival	Higher levels expression correlated with:		
				Higher T-stage,	$P = 0.008$	[91]
				Higher N-stage,	$P = 0.049$	
				Increased risk of tumor recurrence	$P = 0.01$	
				Poor survival	$P < 0.001$	
		20	Survival	Strong staining correlated with poorer survival	$P = 0.03$	[92]
		145	Distant metastasis, local recurrence and survival	Strong staining correlated with:		
				Distant metastasis	$P = 0.02$	[93]
				Local recurrence	$P = 0.05$	
				Poorer survival	$P = 0.002$	
	NF-κB	43	Survival	Activated NF-κB predictive of:		
				Poorer disease free survival	$P = 0.010$	[94]
				Poorer overall survival	$P = 0.015$	
Limitless replicative potential	Telomerase	46	Survival	Higher telomere-length ratio shown to be an independent poor prognostic factor	$P < 0.02$	[95]
Sustained angiogenesis	CD105	75	Survival, angiolymphatic invasion, lymph node metastasis and tumor stage and distant metastasis	Significant correlation between expression and:		
				Poorer survival	$P < 0.01$	[96]
				Presence of angiolymphatic invasion	$P < 0.05$	
				More lymph node metastasis	$P < 0.01$	
				Higher tumor stage	$P < 0.001$	
				More distant metastasis	$P < 0.01$	
	VEGF	75	Survival, angiolymphatic invasion, lymph node metastasis, stage of tumor and distant metastasis	Significant correlation between high expression and:		
				Poorer survival	$P < 0.01$	[96]
				Presence of angiolymphatic invasion	$P < 0.05$	
				More lymph node metastasis	$P < 0.01$	
				Higher stage of tumor	$P < 0.01$	
				More distant metastasis	$P < 0.01$	
Tissue invasion and metastasis	Cadherin	59	Survival	Reduced level correlated with poorer overall survival	HR: 3.3, $P = 0.05$	[80]
	uPA	54	Survival	High uPA correlated with poorer survival	$P = 0.0002$	[97]
	TIMP	24	Survival and disease stage	Reduction of expression correlated with poorer overall survival and higher disease stage	$P = 0.007$	[98]
					$P = 0.046$	
Others	Promoter hypermethylation	41	Survival and tumor recurrence	Earlier tumor recurrence and poorer overall survival if > 50% of gene profile methylated	$P = 0.05$	[99]
					$P = 0.04$	
		84	Differentiation	Hypermethylation of MGMT (Methylated-DNA-protein-cysteine methyltransferase) gene correlated with:	$P = 0.0079$	[100]
				Higher tumor differentiation		

EGFR: Epidermal growth factor receptor; Her2/neu: Human EGFR2; TGF: Transforming growth factor; APC: Adenomatosis polyposis coli; P21: Cyclin-dependent kinase inhibitor 1; Bcl-2: B-cell lymphoma 2; COX-2: Cyclooxygenase-2; NF-κB: Nuclear factor-κB; CD105: Endoglin; VEGF: Vascular endothelial growth factor; uPA: Urokinase-type plasminogen activator; TIMP: Tissue inhibitor of metalloproteinase.

specificity. Panels of biomarkers such as the eight-gene methylation panel or the Reid panel, which combine LOH at various loci and DNA content abnormalities to predict progression, seem to provide the most accurate predictor of progression based on statistics. Unfortunately, the common theme in these panels of markers is

Table 3 Summary of the molecular signatures discovered by microarray technology and latest methods used to correlate molecular alterations and prognosis in patients with esophageal adenocarcinoma

Method	Sample size (*n*)	Outcome	Findings	Statistical significance	External validation	Ref.
Oligonucleotide cRNA microarray	75	Survival	A 4-gene signature prognosticated patients	$P = 0.0001$	Yes	[113]
	77	Lymphatic spread	Created a gene signature predicting lymph node metastasis	Argininosuccinate synthetase expression (ASS) (P = 0.048)	No	[114]
	19	Chemotherapy response	Unsupervised hierarchical clustering divided patients into 2 groups, one of which responded to preoperative chemotherapy	Not statistically significant	No	[115,116]
	47	Chemotherapy response	86 genes dysregulated Ephrin B3 expression associated with chemotherapy response, tumor grading and stage	$P < 0.001$	No	[117]
Oligonucleotide cDNA microarray	46	Chemotherapy response	Gene signature not predictive in adenocarcinoma of esophagus	Not statistically significant	No	[118]
Proteomic analysis	34	Chemotherapy response	HSP27 expression associated with response to chemotherapy	$P < 0.05$	No	[119]
Single nucleotide polymorphism	210	Survival and recurrence	5 polymorphisms in 3 genes associated with longer recurrence free survival and reduced recurrence	$P = 0.004$	No	[120]
microRNAs analysis	96	Survival	Low miR-375 levels associated with worse survival	$P = 0.002$	No	[121]
Multiplex ligation-dependent probe amplification	33	Survival	Patients with more than 12 chromosomal aberrations had a poorer outcome than patients with < 12	$P = 0.014$	No	[122]

that they are far too expensive to be applied in routine clinical use, and technical expertise is not available in all centers to utilize these panels of biomarkers. The issue of costs and practicality of biomarkers should be one of the principle considerations before research and resources are channeled into it.

Although traditional methods of identifying biomarkers in Barrett's esophagus and its transition to dysplasia and EA have helped greatly in the understanding of the disease process, new technologies to create molecular signatures have also helped by identifying many important biomarkers not previously thought to be involved in its pathogenesis. A few biomarkers identified from both traditional methods and new technological platforms have shown great potential in predicting the progression from Barrett's esophagus to EA. However, a concerted effort is still needed to validate these biomarkers or molecular signatures in independent, large-scale prospective cohorts and to develop inexpensive, practical assays to allow for clinical applicability. Realistically, this can only be achieved by a multicenter collaboration to tackle the challenges of the large amount of resources, scientific and clinical input required to advance the field of biomarkers in Barrett's esophagus. There are a few major collaborations in the United Kingdom to date, and they include the Chemoprevention of Premalignant Intestinal neoplasia trial (CHOPIN) and Oesophageal Cancer Clinical and Molecular Stratification Study (OCCAMS). This is also mirrored in the international arena with Barrett's Esophagus and Adenocarcinoma Consortium (BEACON) and Asian Barrett's Consortium as two examples of collaborative work on Barrett's esophagus. These initiatives allow for the pooling of resources, expertise and knowledge between centers and allow for the recruitment of large numbers of patients that are necessary to advance the field of biomarkers in Barrett's esophagus and EA. Although each study has a slightly different focus, much could be gained these collaborative efforts if a proportion of the resources and patient samples could be used to validate biomarkers in Barrett's or tumor samples.

Lastly, biomarkers should be seen as adjuncts to aid clinical management of patients with Barrett's esophagus and EA rather than in isolation in predicting the risk of progression, prognosis or response to therapy. As such, clinical factors in conjunction with biomarkers should be incorporated into a model that can accurately determine the desired outcome. Such models have been used in other cancers and diseases such as the MELD score for liver disease or the Nottingham prognostic index for breast cancer. Upon generation and validation of the model, it should then be rigorously validated in an independent large cohort of patients in a prospective fashion. In future, patients can then be risk stratified based on a score to determine the treatment strategy, hence individualizing treatment to improve patient care and outcome.

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REVIEW

Management of mucinous cystic neoplasms of the pancreas

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Abstract

The purpose of this study was to investigate the actual management of mucinous cystic neoplasm (MCN) of the pancreas. A systematic review was performed in December 2009 by consulting PubMed MEDLINE for publications and matching the key words "pancreatic mucinous cystic neoplasm", "pancreatic mucinous cystic tumour", "pancreatic mucinous cystic mass", "pancreatic cyst", and "pancreatic cystic neoplasm" to identify English language articles describing the diagnosis and treatment of the mucinous cystic neoplasm of the pancreas. In total, 16322 references ranging from January 1969 to December 2009 were analysed and 77 articles were identified. No articles published before 1996 were selected because MCNs were not previously considered to be a completely autonomous disease. Definition, epidemiology, anatomopathological findings, clinical presentation, preoperative evaluation, treatment and prognosis were reviewed. MCNs are pancreatic mucin-producing cysts with a distinctive ovarian-type stroma localized in the body-tail of the gland and occurring in middle-aged females. The majority of MCNs are slow growing and asymptomatic. The prevalence of invasive carcinoma varies between 6% and 55%. Preoperative diagnosis depends on a combination of clinical features, tumor markers, computed tomography (CT), magnetic resonance imaging, endoscopic ultrasound with cyst fluid analysis, and positron emission tomography-CT. Surgery is indicated for all MCNs.

Key words: Pancreatic cystic lesion; Pancreatic mucinous cystic neoplasm; Pancreatic mucin-producing cysts; Pancreatic cystic neoplasm; Pancreatic ovarian-type stroma

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INTRODUCTION

Becourt first described cystic lesions of the pancreas in 1824[1]. In 1978, Compagno *et al*[2] first classified cystic tumors into serous cystic neoplasms (SCNs) and mucinous cystic neoplasms (MCNs) of the pancreas and identified MCN as a distinct disease occurring almost exclusively in the pancreas body and tail of middle-aged women[2,3]. Until 1996, when the World Health Organization distinguished between intraductal papillary mucinous neoplasms (IPMNs) and MCNs, emphasizing the presence of ovarian stroma in the latter, and until 1997 when the Armed Forces Institute of Pathology confirmed this distinction, MCN and IPMNs were frequently confused[3-7]. Nowadays, they represent two distinct neoplasms with different biologic behaviour, pathologic features, and prognosis[8-11].

Although until 1987, Warshaw *et al*[12] considered that pseudocysts account for the majority of pancreatic cys-

tic lesions, nowadays mucinous and serous cystic tumors represent 50%-60% of all cystic lesions[13]. Nevertheless pancreatic cystic neoplasms occur with less frequency than solid ones[4,14,15], but are now found with increasing frequency compared to the past due to the improvement and refining of modern imaging techniques like multidetector, three-dimensional computed tomography (CT) or magnetic resonance imaging (MRI), or endoscopic ultrasound (EUS)[16].

The aim of this study was to review the literature to clarify the management of cystic mucinous neoplasm of the pancreas.

LITERATURE SEARCH

A comprehensive literature review was performed in December 2009 by consulting PubMed MEDLINE for publications, matching the key words of "pancreatic mucinous cystic neoplasm", "pancreatic mucinous cystic tumor", "pancreatic mucinous cystic mass", "pancreatic cyst" and "pancreatic cystic neoplasm" to identify English language articles on MCNs.

Only studies including series with more than four patients affected by MCNs were included. Articles reporting reviews, case reports, abstracts and studies on only IPMNs, SCNs or pancreatic pseudocysts were excluded. Definition, epidemiology, anatomopathological findings, clinical presentation, preoperative evaluation, treatment and prognosis were analyzed.

A total of 16 322 references ranging from January 1969 to December 2009 were analyzed ("pancreatic mucinous cystic neoplasm", n = 930; "pancreatic mucinous cystic tumor", n = 924; "pancreatic mucinous cystic mass", n = 143; "pancreatic cyst", n = 6215; "pancreatic cystic neoplasm", n = 8110) and 77 articles were selected[10,14,17-89]. No articles before 1996 were usable because MCNs were not previously considered as a completely autonomous disease[9-11,17,77,86,89,90].

DEFINITION AND EPIDEMIOLOGY

MCNs are defined as mucin-producing and septated cyst-forming epithelial neoplasia of the pancreas with a distinctive ovarian-type stroma. Usually solitary, their size ranges between 5 and 35 cm with a thick fibrotic wall and without communication with the ductal system[11]. MCNs are rare and, in most series, less common than IPMNs and SCNs[73]. MCNs show a female to male ratio of 20 to 1 and a mean age at diagnosis of between 40 and 50 years (range 14-95 years)[6,7,10,11,91-93]. The site of the neoplasm is in the body and tail of the pancreas in 95%-98% of cases[3,7,9,34,35,89,94,95]. When localized in the pancreatic head, mucinous cystoadenocarcinoma is more prevalent[7,10].

Invasive carcinoma incidence in MCN varies between 6% and 36%[8-11,14,34,35,86]. The Ulm series reported on 39 patients with MCNs and a malignant histology in 51%, including carcinoma *in situ* and advanced cancer[11]. The explanation of this wide range may be the difficulty in interpreting the data on the prevalence of carcinoma because the majority of series have only indicated the advanced form.

ANATOMOPATHOLOGICAL FINDINGS

Macroscopically, MCNs usually appear as solitary, multilocular or unilocular lesions with a mean size of 7-8 cm (range 0.5-35 cm) with a thick fibrotic wall and containing mucin, even when hemorrhagic, watery or necrotic content is observed[8].

In 2004, the consensus conference of the International Association of Pancreatology in Sendai (Japan)[8,9] established that the histological presence of unique ovarian-type stroma was mandatory to diagnose MCN and that this was not found in other pancreatic neoplasms[10,73,93]. MCNs display no communication with the pancreatic ductal system, although some studies suggested that a small proportion of MCNs may show microscopic communication with the pancreatic ducts[68,96,97].

Under light microscopy, the cysts are lined by a columnar mucin-producing epithelium with different grade of dysplasia: mild (MCN adenoma), moderate (MCN borderline), and severe (MCN carcinoma *in situ*)[98]. The epithelial lining is positive for CKs (CK7, CK8, CK18, CK19), EMA and, less frequently, CK20, CEA, DUPAN-2 and CA 19-9[8,10,67]. An invasive adenocarcinoma of the tubular or ductal type is associated in about one-third of cases[6]. The immunophenotype of ovarian-type stroma is similar to the normal ovarian one with positivity for vimentin, calretinin, tyrosine hydroxylase, SMA, α-inhibin, Melan-A, CD99 and Bcl-2 and frequently for PR and ER. The origin of ovarian stroma of the pancreas is still being debated[99]. A stimulation of endodermal immature stroma by female hormones or primary yolk cell implantation in the pancreas has been suggested in literature[10] because buds of the genital tract and dorsal pancreas are adjacent to each other during embryogenesis. Moreover, dorsal pancreatic enlargement mainly gives rise to the pancreatic body and tail, and this could explain the predilection of MCNs for the distal pancreas[17].

Although the pathologic diagnosis of malignancy is based on invasion of the pancreatic parenchyma or metastases[5], MCNs that do not have conclusive evidence of carcinoma are considered premalignant[7].

A thickened wall with peripheral calcification and papillary proliferations, vascular involvement and hypervascular pattern should be considered as suggestive of MCN with malignant changes[68,95]. Although the invasive MCN (mucinouscystoadenocarcinoma or mucinous cystic neoplasm with associated invasive carcinoma) is generally a tubular/ductal carcinoma[8], rare histological variants are represented by undifferentiated carcinoma with osteoclast-like giant cells[100], adenosquamous or colloid cells[101], or sarcomatoid carcinoma[99], carcinosarcoma and choriocarcinoma[8,102].

The increasing degree of dysplasia and tendency for invasion have been correlated with activating point mutations in the *k-ras* gene and mutations in the *TP5* gene[8,103,104]; moreover, the discovery that the inactiva-

tion of SMAD4/DPC4 in the epithelium of the invasive MCNs, but not in the ovarian-like stroma, could suggest that the ovarian-type stroma is not neoplastic[105].

CLINICAL PRESENTATION

The majority of MCNs are slow growing and asymptomatic[95]. In a series of 212 consecutive patients with cystic pancreatic lesions, 36.7% were asymptomatic and among them 28% had MCNs; in the symptomatic group, 16% had MCN[106]. In spite of these lesions being occasionally discovered in patients scanned for other indications[16,35,107], the typical clinical appearance is characterized by epigastric heaviness and fullness (60%-90%) or by an abdominal mass (30%-60%)[7,10,12,35,89,106,108]. Nausea, vomiting (20%-30%) and back pain (7%-40%) can also be present.

No specific symptom was significantly associated with a likelihood of malignancy[35] although increasing anorexia and weight loss (10%-40%) may be associated with malignant changes[7,12,35,89,95,106,108,109].

PREOPERATIVE EVALUATION

MCNs main differential diagnosis includes other neoplastic cystic lesions (serous cystic neoplasm and the intraductal papillary mucinous neoplasms) and non-neoplastic cystic lesions (pancreatic pseudocysts). There is no single discriminating test, but preoperative diagnosis depends on a combination of modes, including clinical features, tumor markers, CT and MRI, EUS with cyst fluid analysis, and positron emission tomography (PET).

High values of CEA and CA 19-9 show a high positive predictive value for pancreatic malignancy or premalignancy in the preoperative assessment of pancreatic cystic mass (70%-100%)[9,95,110]. A CEA level of more than 400 ng/mL is a good predictor of malignancy in MCNs (sensitivity 45%-50%, specificity 95%-100%, accuracy 75%-80%)[107,111,112].

Trans-abdominal ultrasound examination has a low accuracy (50%) for cystic neoplasms of the pancreas[89].

EUS improves that accuracy and allows better evaluation of the wall as it may show separation or nodules within the cyst. Furthermore, EUS can be used to obtain aspiration of the cyst contents and to perform a biopsy of the wall. Cyst fluid amylase concentration of < 250 U/L has been considered capable of excluding pseudocysts of the pancreas (sensitivity 40%-45%, specificity 95%-100%, accuracy 60%-65%), while CEA < 5 ng/mL could suggest a benign etiology (sensitivity 45%-50%, specificity 95%-100%, accuracy 65%-70%)[31,111]. EUS-FNA cytology and cyst fluid CEA greater than 192 ng/mL show the highest accuracy (79%) for differentiating mucinous cystic from non-mucinous cystic neoplasms[113]. On the contrary EUS morphology alone cannot distinguish between the two groups[47,50,113].

In any case, the main differential diagnosis of MCNs is with SCNs which have a low CEA in the fluid and an equal distribution throughout the pancreas, with pancreatic pseudocysts (PC) that usually show necrotic debris within the cyst cavity, and with branch duct IPMNs communicating with the ductal pancreatic system and consequently showing elevated cystic fluid amylase[3].

Although pancreatitis may be present in the history of patients with pancreatic cystic neoplasms, when a cyst arises in a patient with chronic pancreatitis, the most frequent diagnosis is PC[109]. On the other hand, when pancreatitis is unexpected and occurs for the first time, the cyst could be a tumor, determining the development of pancreatitis due to compression of the pancreatic duct[13]. This is a crucial problem, because the risk of managing cystic mucinous neoplasms in patients with a prior history of pancreatitis, like pseudocysts by a pseudocyst-jejunal anastomosis or pseudocyst-gastrostomy, is higher than usual, with disastrous long-term prognosis[12,83]. Proper sampling of pseudocysts is essential and should consist of sampling of the cyst wall during surgery or analysis of cyst content during minimal access drainage procedures. Although the clinical context, radiological imaging and biochemical findings may help differentiate PC from cystic neoplasms, small lesions may be problematic.

The image based classification system proposed by Sahani *et al*[107], in which cystic pancreatic lesions are classified in four subtypes, is reported in Table 1.

The demonstration of a solid component, invasion outside the confines of the pancreas, or pancreatic duct obstruction through EUS is highly indicative of malignancy with sensitivity, specificity and accuracy of 70%, 100% and 60%, respectively[107]. However, in the absence of these findings the ability of EUS to diagnose malignancy is limited with an overall sensitivity, specificity and accuracy of 56%, 45% and 51%, respectively[113]. The added advantage of EUS in performing aspiration of cyst content and sampling of the cyst wall and septa or mural nodules is that it allows small lesions as well as suspicious areas to be analysed. Laparoscopic and intraoperative ultrasounds are highly operator dependent with an accuracy

Table 1 Imaging-based classification system of cystic pancreatic lesions

Type of lesions	Morphologic features	Pancreatic cystic lesions
Unilocular cyst	Without internal septation and solid component or wall calcification	Serous cystic neoplasm, intraductal papillary mucinous neoplasm[1], pancreatic pseudocyst
Microcystic lesion	Six or more cysts with diameter 0.2 mm-2 cm, external lobulation, fibrous central scar with or without stellate calcifications	Serous cystic neoplasm
Macrocystic lesions	Diameter > 2 cm, with internal septation and solid component or wall calcification	Intraductal papillary mucinous neoplasm[1], mucinous cystic neoplasm[2]
Cyst with a solid component	Unilocular or multilocular	Intraductal papillary mucinous neoplasm[1], mucinous cystic neoplasm[2]

[1]With or [2]without communication with main duct, respectively.

ranging from 40% to 90%[3,114-120].

Multidetector computed tomography and magnetic resonance cholangiopancreaticography (MRCP) play a critical role in assessment, defining size, septation, calcifications, nodules of the wall, and communication with the ductal system of the pancreatic cyst.

At cross-sectional imaging, the MCN appears as a unilocular or multilocular single macrocyst with a solid component, with no communication with the main duct[95,107,121]. The internal architecture of the cyst, including septa and internal wall, is best appreciated with MR imaging[122,123].

Recently, Kim *et al*[60] defined some significant CT features for differentiating MCNs from SCNs and IPMNs: the shape is smooth in MCNs, multicystic and lobulated in SCNs, and pleomorphic and clubbed finger-like in IPMNs; the main pancreatic duct is not dilated or proximally only in SCNs, and if dilated, whole in IPMN.

In spite of the improvement in pancreatic tumor visualization resulting from CT and MRI, the ability to perform diagnosis of these techniques individually - as well as EUS - remains poor (25%-30%)[6,124,125]. In a multivariate analysis by Visser *et al*[14] in 2008, the combination of CT and MRI data showed an accuracy ranging from 44% to 83%.

Cross-sectional imaging generally shows peripheral calcification, a thickened wall, papillary proliferations, vascular involvement and hypervascular pattern in the cases of malignant MCNs[36,95,126]. Although peripheral eggshell calcification is not easily detected by CT, this is a specific feature of the MCNs and is highly predictive of malignancy[126].

The clinical value of MRCP is similar to endoscopic retrograde cholangiopancreatography or percutaneous transhepatic cholangiography[127] but an MR multi-imaging protocol, which includes MR cross-sectional imaging, MRCP and dynamic contrast-enhanced MR angiography, integrates the advantages of multiple imaging techniques without morbidity[128-131].

The role of PET in managing pancreatic cystic lesions is currently limited but recent studies report detection of malignant pancreatic cysts with sensitivity and positive predictive values above 90%[132,133].

In spite of a complete diagnostic assessment, the surgeon's preoperative diagnosis is correct in one-third of cases, incorrect in another third, and non-specific in the remainder[6,14].

TREATMENT

Surgical excision is indicated for all MCNs considered premalignant. Factors influencing treatment include tumor histological features, the patient's age and surgical risk, and tumor size and location.

Left pancreatectomy

Because mucinous cystic adenoma of the pancreas are usually localized at the level of the body and tail of the pancreas, the most common operation performed to cure these neoplasms is distal pancreatectomy (Figure 1), which is a safe procedure in high volume centres (overall postoperative morbidity ranging from 5% to 50% and a mortality rate of 0%)[3,7,20,35,134,135]. The main complication, pancreatic fistula, occurs in 15%-20% of cases[136].

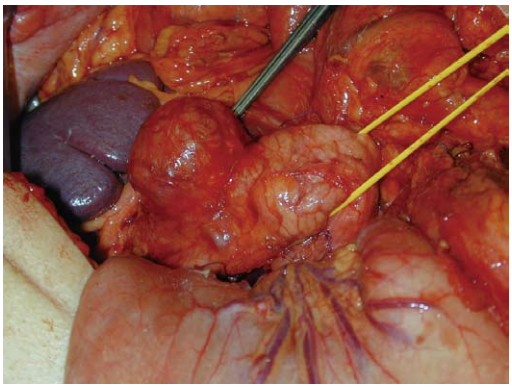

Figure 1 Distal pancreatectomy. Dissection of the pancreas in a patient with mucinous cystic neoplasm.

The distal pancreatectomy technique was first described in 1913 by Mayo[137] and the spleen-preserving distal pancreatectomy was outlined in 1943 by Mallet-Guy *et al*[138]. Preservation of the spleen can be performed with or without preservation of the splenic artery and vein. In 1988, Warshaw described a technique without the preservation of the splenic artery and vein, ligating the splenic vessels at the hilum[139]. Although this method appears technically less difficult and can be performed in a shorter operating time, it has been associated with a higher incidence of spleen vascular insufficiency[140]. However, this procedure should be considered in the event of an inflamed or fibrosed splenic artery and vein[139]. Spleen-preserving techniques must be avoided when in the presence of the largest tumors or risk factors for invasive malignancy, such as the size of the lesion, eggshell calcifications and mural nodules, in order to perform the complete oncological lymph node dissection[9,27,31]. However, these techniques are preferred in all other cases to avoid long term infectious and haematological complications[3,20,27,139].

Studies comparing patients undergoing distal pancreatectomy with or without splenectomy show no significant differences compared to perioperative complications, mean operating time, pancreatic fistula rate, length of hospital stay and mortality[20,108,135,141,142].

MCNs affecting the pancreatic neck or the proximal body could be managed either by an extended right or, more frequently, by an extended left pancreatectomy. These extended resections of normal pancreatic tissue may induce endocrine and exocrine insufficiency respectively in 30%-35% and 15%-20%, which in a benign or premalignant disease could be discussable[143-147].

Middle pancreatectomy

Middle pancreatectomy can be considered in the surgical management of MCNs located at the level of the pancreatic proximal body or neck, preserving endocrine and

exocrine function with respect to extended left pancreatectomy or pancreaticoduodenectomy, and also preserving the spleen.

The main pitfalls of this technique are the technical difficulty, the higher incidence of postoperative complications and the risk of recurrence from potentially residual neoplasm[9,146-153].

Different techniques have been proposed for gastrointestinal reconstruction, including jejunal anastomosis of the stump or the distal stump, with pancreaticoduodenal or pancreaticogastric anastomosis[143,148-156].

In the literature, mortality after middle pancreatectomy was none and the overall morbidity was 25%-35%[147-149,155-157]. The incidence of overall pancreatic fistula was 22%-45% and the type of reconstruction through Roux-en-Y pancreatojejunostomy or pancreaticogastrostomy did not affect the rate of any complication[147-149,155-157]. Moreover, the incidence of endocrine and exocrine insufficiency after middle pancreatectomy was 4%-7% and 5%-8%, respectively[147-149,155-157].

Enucleation

Because the probability of malignancy in patients with MCNs smaller than 2 cm without nodules is very low, enucleation could be performed to avoid post-operative pancreatic insufficiency[35]. This procedure is proposed for patients with MCNs smaller than 2 cm with benign features and superficially located[146,155,156]. Enucleation can be performed without risk of recurrence but has been associated with a higher incidence of pancreatic fistula (30%-50%)[158-160].

Whipple procedure

A major oncologic resection, applying a Kausch-Whipple or pylorus-preserving technique, is recommended for MCNs that are localized monocentrically in the head.

The operative mortality ranges from 0% to 5% and is generally related to pancreatic anastomosis complications[136,161]. The most common complications following the Whipple procedure are delayed gastric emptying and the pancreatic fistula occurring in 5%-10% and 6%-20% of operations, respectively[136,161-163].

When an enucleation is impossible or contraindicated, MCNs localized monocentrically in the pancreatic head that do not have an association with an invasive pancreatic cancer could be treated by duodenum-preserving total pancreatic head resection[34,94,164-167].

This procedure shows significant advantages when compared to Traverso-Longmire or Whipple pancreaticoduodenectomy, as regards the postoperative rate of morbidity and mortality, glucose metabolism, hospitalization and costs[11,168].

Lymphadenectomy

Pancreatectomy with lymph node dissection is necessary when an invasive carcinoma is suspected. Although the preoperative and intraoperative assessment of the grade of invasiveness is often difficult, whenever any doubt exists typical resection with lymph node dissection must be pursued[9]. There is no evidence in literature of invasive mucinous cystic adenocarcinoma with distant lymph node metastases, so only a loco-regional lymphadenectomy is justified[3,35]. Because the probability of malignancy is very low in the cases of small MCNs without nodules, lymphadenectomy can be avoided[3,146].

Laparoscopy

In the cases of benign-appearing and small malignant lesions (< 5 cm), a minimally invasive approach may be considered[35]. Recent experiences from high-volume centers demonstrate that the laparoscopic approach for distal pancreatectomy for MCNs of the body and tail of the pancreas is feasible and safe[169]. The complication rate of laparoscopic distal splenopancreatectomy (Lap SDP) ranges between 15% and 20%[30,46,170-173] with a mortality rate of 0%. In spleen-preserving laparoscopic pancreatic (Lap SPDP) resection, the overall morbidity ranges from 25% to 40% with a mortality rate of 0%[30,46,169-173]. The overall reported pancreatic fistula was 5%-8% and 10%-15% after Lap SPDP and Lap SDP, respectively[30,46,170-173]. This laparoscopic approach decreases the hospital stay and minimizes the cosmetic impact of the surgical wound[30,169,174,175].

Chemotherapy

Gemcitabine (GEM) is the standard therapy for advanced pancreatic cancer[176]. Its effectiveness against advanced MCNs has been reported[177,178].

Recently, some combinations have been reported to be superior to GEM alone[177,179-181]. GEM-oxaliplatin treatment has been proposed to be more effective in terms of clinical progression-free survival[177].

Discordant results on survival were reported by phase Ⅱ and Ⅲ trials combining GEM and inhibitors of epidermal growth factor receptor (cetuximab) and vascular endothelial growth factor (bevacizumab)[182-184].

Other modest but interesting advances have been provided by combinations such as GEM-capecitabine and GEM plus a platinum salt[185]. In spite of this, survival results remain disappointing.

Conservative treatment

A conservative management with regular follow-up has been proposed in the presence of asymptomatic cystic lesions of the pancreas smaller than 3 cm without mural nodules, because the reported risk of malignancy in these cysts was found to be 3%[11,35,63,186]. The suggested follow-up consisting of cross-sectional imaging and FNA cytology should be performed every 6 mo for a period of 2 years and yearly after that. This should be continued for at least 4 years and then the interval of follow-up can be lengthened after 6 years of no change[31,52,186]. When the cyst enlarges or when symptoms occur (in up to 20% of patients after follow-up), surgery is mandatory. The reported incidence of the subsequent resection due to change of the clinical, radiological and biochemical features of the lesions after initial conservative treatment was 4%-10% and malignancy rate in these cases was 3%[23,63].

PROGNOSIS

After resection, in the absence of invasive carcinoma, prognosis of MCNs is excellent, with an overall survival rate of 100%[5-7,10,35] and patients do not need follow-up, since several studies have shown that the risk of recurrence following resection is 0%[11,18]. Patients with invasive mucinous cystadenocarcinoma, show a 5-year survival rate of 20%-60%, which is much better than that for non-MCN-associated ductal adenocarcinoma[5-8,11,17,35,73,86]. When an anaplastic carcinoma of the pancreas associated with MCN is reported, the prognosis is obviously extremely poor, with a 3-year survival rate lower than 3%[5-8,11,17,35,73,86].

CONCLUSION

Although the histological distinction between MCNs and IPMNs, through the identification of ovarian stroma initially, is very important in clinical practice, the management of MCNs has not yet been standardized and continues to evolve.

The approach to patients with suspected MCN is based on EUS and cross-sectional imaging in association with FNA cytology, detecting an incidence of correct differentiation between mucinous cystic and non-mucinous cystic neoplasms of 75%.

Because at present we are unable to identify the benign MCNs that will progress into invasive carcinoma, all MCNs should be resected, regardless of size, in patients who are fit candidates for surgery, because surgery is routinely curative in the cases of non-invasive tumor. Moreover, the non-operative management based on periodic CT or MRI requires years of careful follow-up with a high cost of imaging and the enucleation technique carries the risk of non-oncological radicality. In patients with non-invasive MCN after complete anatomic resection, postoperative surveillance is unnecessary.

In order to obtain more benefit by applying adjuvant chemotherapy for the treatment of the advanced MCNs, further research focused on sequential cellular transformation from benign to malign tumor and on new combinations, incorporating the new targeted therapies and identifying potential predictive factors of response, is required to be able to offer effective tailored treatment to these patients.

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ORIGINAL ARTICLE

Insulin like growth factor-1 increases fatty liver preservation in IGL-1 solution

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Abstract

AIM: To investigate the benefits of insulin like growth factor-1 (IGF-1) supplementation to serum-free institut georges lopez-1 (IGL-1)® solution to protect fatty liver against cold ischemia reperfusion injury.

METHODS: Steatotic livers were preserved for 24 h in IGL-1® solution supplemented with or without IGF-1 and then perfused "*ex vivo*" for 2 h at 37℃. We examined the effects of IGF-1 on hepatic damage and function (transaminases, percentage of sulfobromophthalein clearance in bile and vascular resistance). We also studied other factors associated with the poor tolerance of fatty livers to cold ischemia reperfusion injury such as mitochondrial damage, oxidative stress, nitric oxide, tumor necrosis factor-α (TNF-α) and mitogen-activated protein kinases.

RESULTS: Steatotic livers preserved in IGL-1® solution supplemented with IGF-1 showed lower transaminase levels, increased bile clearance and a reduction in vascular resistance when compared to those preserved in IGL-1® solution alone. These benefits are mediated by activation of AKT and constitutive endothelial nitric oxide synthase (eNOS), as well as the inhibition of inflammatory cytokines such as TNF-α. Mitochondrial damage and oxidative stress were also prevented.

CONCLUSION: IGL-1® enrichment with IGF-1 increased fatty liver graft preservation through AKT and eNOS activation, and prevented TNF-α release during normothermic reperfusion.

Key words: AKT; Institut georges lopez-1® solution; Insulin like growth factor-1; Ischemia reperfusion injury; Nitric oxide; Oxidative stress; Steatotic graft preservation

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INTRODUCTION

One of the major challenges in liver transplantation is to increase the use of marginal organs. Steatotic, or fatty, livers are increasingly transplanted, in spite of the associated risk of graft dysfunction or non-function as a result of cold ischemia reperfusion injury (IRI)[1,2]. Steatotic livers show poor tolerance to IRI due to severe mitochondrial damage, impaired energy metabolism[3], increased reactive oxygen species (ROS) and release of inflammatory cytokines such as tumour necrosis factor-α (TNF-α), which impairs microcirculation[4,5]. All these factors render steatotic livers more vulnerable to cold IRI.

Liver preservation is a crucial step in maintaining graft quality after prolonged ischemic periods, especially steatotic livers. University of Wisconsin (UW) is the most widely used serum-free preservation solution for transplantation but it does not fully protect liver grafts during prolonged storage[6-8]. Recently, the new institut georges lopez-1 (IGL-1)® solution has been proposed as an effective alternative to UW in clinical kidney transplantation[9,10], and in experimental orthotopic liver transplantation models[11,12]. IGL-1® solution is characterized by inversion of K^+ and Na^+ concentrations in the UW solution and contains polyethylene glycol as osmotic support instead of HES. In addition, we have previously demonstrated that IGL-1® is more suitable than UW solution for fatty liver preservation[13]. Its benefits are associated in part, with the prevention of oxidative stress and its capacity to generate nitric oxide (NO)[13]. NO is a vasodilator with anti-inflammatory properties that prevents microcirculatory alterations[5,13] and the release of pro-inflammatory cytokines, such as TNF-α, during IRI[14-16].

Insulin like growth factor-1 (IGF-1) is a vascular protective factor that is mainly synthesized and released by the liver[17,18]. We have recently reported impaired synthesis of IGF-1 in steatotic livers subjected to warm IRI, and that exogenous administration of recombinant IGF-1 reduced IRI in steatotic livers[18]. IGF-1 prevents oxidative stress[17] and induces NO generation by eNOS activation, as a consequence of AKT kinase phosphorylation[19,20].

Several trophic factors (TF), including IGF-1, have been added to UW solution in an attempt to improve the survival of pig orthotopic liver allografts after 18 h of cold storage[21]. In vascular endothelial cells, the benefits of TF supplementation are associated with limitation of mitogen-activated protein kinase (MAPK) activities after cold ischemia/rewarming injury[22].

Taking this into account, we explored the effects of the addition of IGF-1 to IGL-1® solution on fatty liver preservation during cold IRI. We examined the mechanisms responsible for such effects, including AKT phosphorylation and NO generation, and the prevention of ROS production and TNF-α release after reperfusion. These latter factors have been implicated in the poor tolerance of steatotic livers to cold IRI.

MATERIALS AND METHODS

Animals and liver procurement

Isolated perfused rat liver was used to evaluate hepatic function in isolation from the influence of other organ systems (undefined plasma constituents and neural/hormonal effects). Hepatic architecture, microcirculation and bile production were preserved in this experimental model, as previously reported[23]. Homozygous obese (Ob) Zucker rats, aged 16-18 wk were purchased from Iffa-Credo (L'Abresle, France) and were housed at 22℃[23]. All procedures were performed under isoflurane inhalation anaesthesia. Experiments were conducted according to European Union regulations for animal experiments (Directive 86/609 CEE).

Liver procurement and experimental groups

The surgical technique was performed as previously reported[23,24]. After cannulation of the common bile duct, the portal vein was isolated and the splenic and gastroduodenal veins were ligated. All animals were randomly distributed into groups as described below. The steatotic livers were flushed and preserved in cold IGL-1® solution for 24 h with or without the addition of IGF-1 (10 μg/L), as described elsewhere[21,22,25].

All animals were randomized according to the experimental protocols, as follows.

Cold storage: After 24 h of cold storage at 4℃, livers from 8 Zucker rats preserved in IGL-1 solution with (IGL-1 + IGF-1) or without IGF-1 were flushed with Ringer's lactate solution. Control livers (Cont 1) from 8 Zucker rats (Ob) were flushed with Ringer's lactate solution at room temperature immediately after laparotomy *via* the portal vein without cold storage. Aliquots of the effluent flush were sampled for measurements of cumulative AST and ALT after 24 h cold storage.

Cold storage and reperfusion: Briefly, after 24 h cold storage at 4℃, livers from 8 Zucker rats (Ob) preserved in IGL-1 and IGL-1 + IGF-1 solutions were subjected to 2 h reperfusion at 37℃, as previously reported[13,24]. Control livers (Cont 2) from 8 Zucker rats (Ob) were flushed with Ringer's lactate at room temperature and immediately perfused *ex vivo* without ischemic preservation.

Transaminase assay

Hepatic injury was assessed in terms of transaminase levels with commercial kits from RAL (Barcelona, Spain)[26].

Briefly, 200 μL of effluent perfusate were added to the substrate provided by the commercial kit and then alanine aminotransferase (ALT)/aspartate aminotransferase (AST) levels were determined at 365 nm with a UV spectrometer and calculated following the supplier's instructions.

Hepatic clearance

As with bile output, hepatic clearance was considered as another parameter of hepatic function. Thirty minutes after the onset of perfusion (t_{30}), 1 mg of sulfobromophthalein (BSP) (Sigma, Madrid, Spain) was added to the perfusate. The concentration of BSP in bile samples (t_{120}) was measured at 580 nm with a UV-visible spectrometer. Bile BSP excretion was expressed as a percentage of perfusate content (t_{120} bile/t_{30} perfusate*100)[23].

Vascular resistance

Liver circulation was assessed by measuring perfusion flow rate and vascular resistance. Perfusion flow rate was assessed continuously throughout the reperfusion period and expressed as mL/min per gram of liver. Vascular resistance was defined as the ratio of portal venous pressure to flow rate and expressed in mmHg/min per gram of liver/mL[26].

Glutamate dehydrogenase activity

Glutamate dehydrogenase (GLDH) was used as an indirect measure of mitochondrial damage. GLDH was measured in the perfusate as described elsewhere[26].

Lipid peroxidation assay

Lipid peroxidation in liver was used as an indirect measure of the oxidative injury induced by ROS. Lipid peroxidation was determined by measuring the formation of malondialdehyde (MDA) with the thiobarbiturate reaction[13].

Determination of nitrite and nitrate

NO production in liver was determined by tissue accumulation of nitrite and nitrate[13].

Western blotting analysis of eNOS, AKT, P38 and ERK 1/2

Liver tissue was homogenized as previously described and proteins were separated by SDS-PAGE and transferred to PVDF membranes[27-29]. Membranes were immunoblotted using the following antibodies: eNOS (Transduction Laboratories, Lexington, KY, USA), total and phosphorylated AKT, total and phosphorylated P38, and total and phosphorylated ERK 1/2 (Cell Signaling, Beverly, MA, USA) and β-actin (Sigma Chemical, St. Louis, MO, USA). Signals were detected by the enhanced chemiluminescence kit (Bio-Rad Laboratories, Hercules, CA, USA) and quantified by scanning densitometry as previously described[26,28].

TNF-α determination

Perfusate TNF-α levels were measured after 120 min of reperfusion using a commercial immunoassay kit for rat TNF-α from Biosource (Camarillo, CA, USA)[30,31].

Statistical analysis

Data are expressed as means ± SE, and were compared statistically by variance analysis, followed by the Student-Newman-Keuls test (Graph Pad Prism software). $P < 0.05$ was considered significant.

RESULTS

Protective effect of IGF-1 against liver injury and function

As shown in Figure 1A and B, the AST and ALT levels observed for steatotic livers preserved for 24 h at 4℃ in IGL-1® solution confirmed their poor tolerance to cold ischemia injury. IGF-1 addition prevented AST/ALT release from steatotic livers when compared with IGL-1 alone.

Similar AST/ALT profiles were obtained when fatty livers were subjected to normothermic reperfusion for 30 and 120 min, respectively. Reduced AST/ALT levels were observed when IGL-1 was enriched with IGF-1 (Figure 1C and D). Similar profiles were seen for 60 and 90 min of reperfusion (data not shown).

The mechanisms by which IGF-1 protects fatty livers against the harmful consequences of cold I/R damage were also investigated. For this reason, liver function was evaluated by the alterations in BSP clearance and vascular resistance, respectively. A reduction in BSP clearance was found in IGL-1 solution when compared to Cont 2. The highest BSP clearance in bile was observed after 2 h of normothermic reperfusion when IGF-1 was present (Figure 2A).

Vascular resistance was increased by IGL-1 solution (Figure 2B), but this was reversed by the addition of IGF-1. These alterations in vascular resistance were also seen in all groups at 30 and 120 min of reperfusion (Figure 2B).

Beneficial effects of IGF-1 on AKT and NO after reperfusion

In order to explore whether the beneficial effects of IGF-1 in IGL-1 solution are associated with other protective cell signalling involved in the protective mechanisms against IRI, we evaluated the changes in AKT and NO levels. As shown in Figure 3, IGF-1 increased AKT phosphorylation when compared to IGL-1 alone and Cont 2, respectively (Figure 3A). This was concomitant with a significant generation of NO, as revealed by the nitrites/nitrates levels, as well as eNOS activation (Figure 3B and C).

Role of IGF-1 on liver oxidative stress, mitochondrial damage and TNF-α release after reperfusion

In order to examine the relationship between the parameters studied and others associated with the poor tolerance of fatty livers to reperfusion injury, we evaluated lipid peroxidation (MDA) and mitochondrial damage (GLDH). Steatotic livers preserved in IGL-1 showed significant MDA augments when compared to Cont 2. These increases were significantly prevented by the addition of IGF-1 (Figure 4A). In accordance with this, IGF-1 also diminished liver mitochondrial damage, as shown by the reduction in GLDH (Figure 4B). These beneficial effects of IGF-1 were

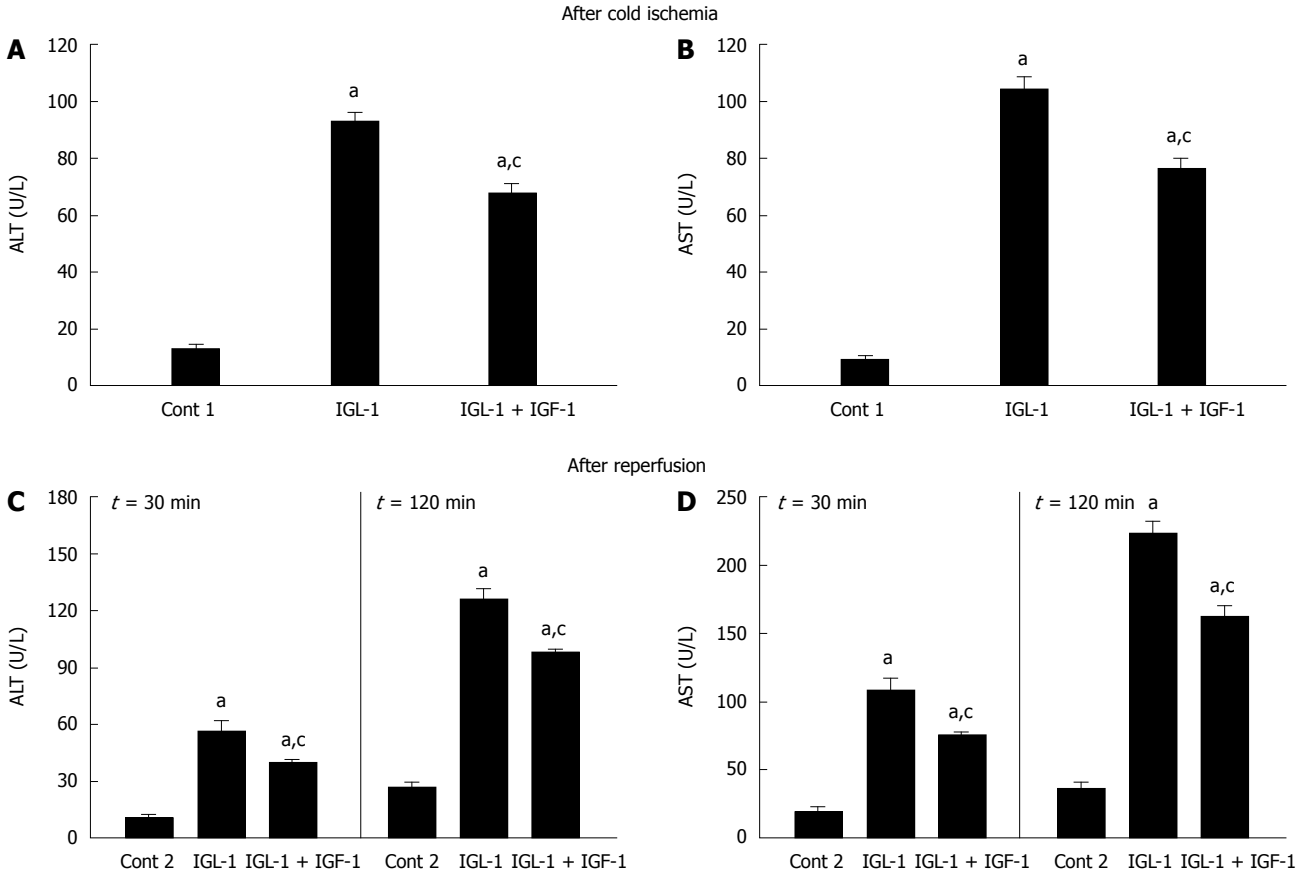

Figure 1 Alanine aminotransferase (A) and aspartate aminotransferase (B) levels in perfusate after 24 h cold storage, alanine aminotransferase (C) and aspartate aminotransferase (D) levels in perfusate after 30 and 120 min of normothermic reperfusion. Cont 1: Liver flushed without cold preservation; IGL-1: Livers preserved in IGL-1 solution; IGL-1 + IGF-1: Livers preserved in IGL-1 with IGF-1. $^{a}P < 0.05$ *vs* Cont 1, $^{c}P < 0.05$ *vs* IGL-1. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IGF-1: Insulin like growth factor-1; IGL-1: Institut georges lopez-1.

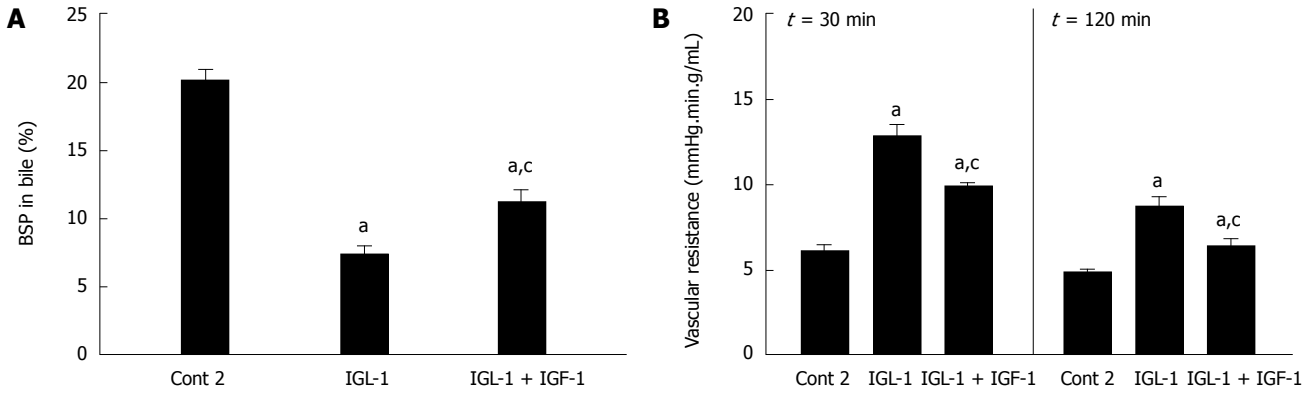

Figure 2 Percentage of sulfobromophthalein in bile (A) and vascular resistance (B) of steatotic livers after 30 and 120 min of normothermic reperfusion. Cont 2: Liver flushed and perfused *ex vivo* without cold preservation; IGL-1: Livers preserved in IGL-1 solution; IGL-1 + IGF-1: Livers preserved in IGL-1 with IGF-1. $^{a}P < 0.05$ *vs* Cont 2, $^{c}P < 0.05$ *vs* IGL-1. BSP: Sulfobromophthalein; IGF-1: Insulin like growth factor-1; IGL-1: Institut georges lopez-1.

also accompanied by a significant reduction in pro-inflammatory cytokine TNF-α level when compared to IGL-1 alone after 2 h normothermic reperfusion (Figure 4C).

Effects of IGF-1 on p-P38 and p-ERK MAPKs after reperfusion

Finally, we examined the effects of IGL-1 with and without IGF-1 on P38 and ERK 1/2 MAPKs, whose activation is closely related with hypothermic conditions. Figure 5 shows increased phosphorylation of ERK 1/2 and P38 MAPK in IGL-1 solution when compared to Cont 2, but again this increase was offset by the addition of IGF-1.

DISCUSSION

IGF-1 is a 70-amino-acid polypeptide, mainly synthesized by the liver, with protective effects against IRI in a variety of tissues[32-35], including the liver[18]. We recently reported

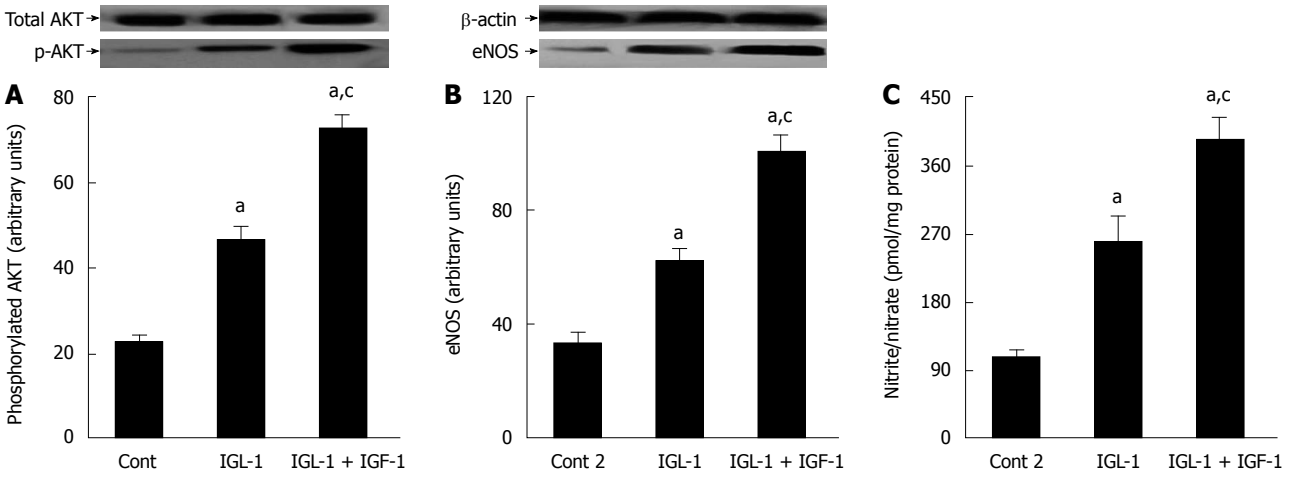

Figure 3 Beneficial effects of insulin like growth factor-1 on AKT and nitric oxide in steatotic liver graft preservation. A: Representative Western blottings of total and p-AKT at the top and densitometric analysis at the bottom after 24 h of cold storage and 120 min of normothermic reperfusion; B: Endothelial nitric oxide synthase (eNOS) protein levels in liver after 120 min of normothermic reperfusion. Representative Western blottings at the top and densitometric analysis at the bottom; C: Nitrite and nitrate levels after 120 min of normothermic reperfusion. Cont 2: Liver flushed and perfused *ex vivo* without cold preservation; IGL-1: Liver preserved in IGL-1 solution; IGL-1 + IGF-1: Livers preserved in IGL-1 with IGF-1. $^{a}P < 0.05$ *vs* Cont 2, $^{c}P < 0.05$ *vs* IGL-1. IGF-1: Insulin like growth factor-1; IGL-1: Institut georges lopez-1.

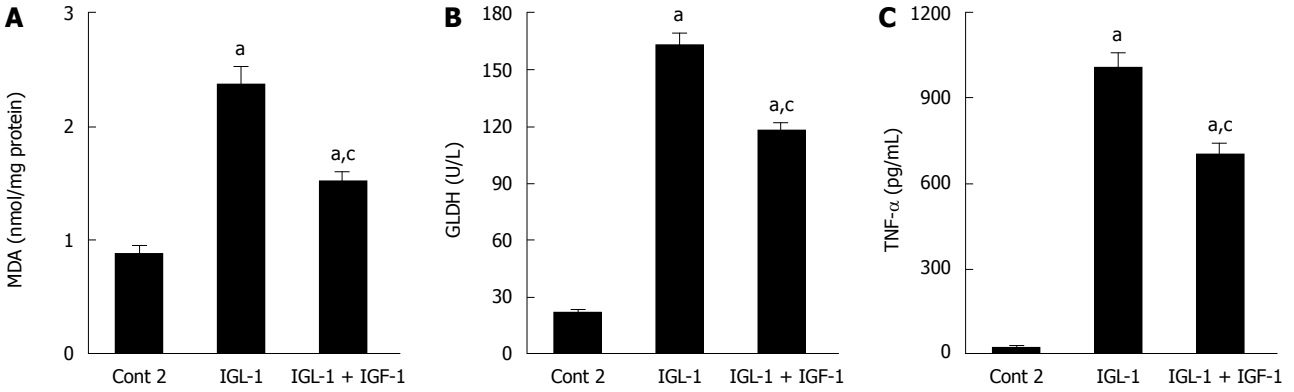

Figure 4 Role of insulin like growth factor-1 on oxidative stress, mitochondrial damage and tumor necrosis factor-α release in steatotic liver. A: Hepatic malondialdehyde (MDA) levels after 120 min of reperfusion; B: Glutamate dehydrogenase (GLDH) activity levels after 120 min of normothermic reperfusion; C: Tumor necrosis factor-α (TNF-α) levels in perfusate after 120 min of normothermic reperfusion. Cont 2: Liver flushed and perfused *ex vivo* without cold preservation; IGL-1: Livers preserved in IGL-1 solution; IGL-1 + IGF-1: Livers preserved in IGL-1 with IGF-1. $^{a}P < 0.05$ *vs* Cont 2, $^{c}P < 0.05$ *vs* IGL-1. IGF-1: Insulin like growth factor-1; IGL-1: Institut georges lopez-1.

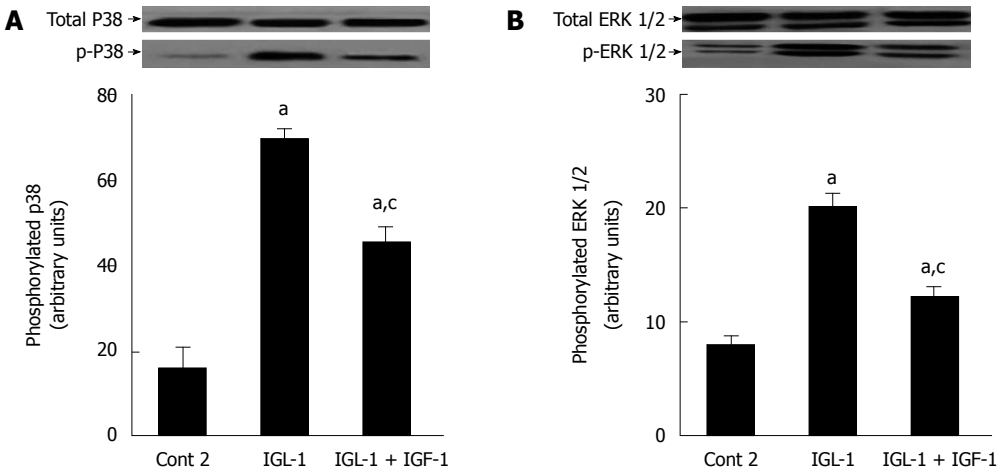

Figure 5 Effects of insulin like growth factor-1 on p-P38 and p-ERK in steatotic livers subjected to cold ischemia reperfusion. A: P38 protein levels in liver after 120 min of normothermic reperfusion. Representative Western blottings of total and p-P38 at the top and densitometric analysis at the bottom after 24 h of cold storage and 120 min of normothermic reperfusion; B: ERK 1/2 protein levels in liver after 120 min of normothermic reperfusion. Representative Western blottings of total and p-ERK 1/2 at the top and densitometric analysis at the bottom. Cont 2: Liver flushed and perfused *ex vivo* without cold preservation; IGL-1: Lliver preserved in IGL-1 solution; IGL-1 + IGF-1: Livers preserved in IGL-1 with IGF-1. $^{a}P < 0.05$ *vs* Cont 2, $^{c}P < 0.05$ *vs* IGL-1. IGF-1: Insulin like growth factor-1; IGL-1: Institut georges lopez-1.

that IGF-1 protects steatotic livers subjected to warm IRI, but its role in fatty liver preservation has been poorly investigated[18]. For this reason, we explored the effect of IGF-1 supplementation of serum free IGL-1® solution, which has been shown to protect fatty livers against cold IRI[13]. We used the concentration of IGF-I (10 μg/L) according to previous studies carried out in different experimental models. In these studies, IGF-I it was used to supplement UW solution in dog kidney transplantation, as well as, primary canine kidney tubule and human umbilical vein endothelial cell cultures[21,22,25,36]. The results reported here show that the addition of IGF-1 to IGL-1® solution significantly improved fatty liver preservation, as evidenced by the reduction in AST/ALT levels. These results are consistent with previous reports by other authors, who demonstrated that the addition of TF to UW solution improves the survival of orthotopic liver allografts[21].

BSP clearance in bile is a useful tool for assessing liver function after prolonged cold ischemia[23,24]. It is well established that complications in biliary structures appear in more than 25% of liver transplant recipients. The increases in BSP clearance observed in IGL-1 + IGF-1 solution revealed that IGF-1 supplementation enhances steatotic graft function.

Steatosis is the result of intracytoplasmic fat accumulation, which is associated with an increase in hepatocellular volume, induced distortion and narrowing of sinusoids with a reduction in the luminal diameter by up to 50% when compared to normal liver[5]. This provokes severe alterations in hepatic blood flow and microcirculation, and prevents appropriate revascularization of the graft. The benefits of the use of IGL-1® solution for fatty liver preservation are associated with its capacity to generate NO[13], a potent vasodilator involved in the regulation of hepatic microcirculation, through eNOS activation, as previously reported by us[24]. Moreover, it has been established that IGF-1 up-regulates eNOS activity by interacting with a tyrosine kinase membrane receptor which activates the AKT signalling pathway[19,37,38]. The results of the present study show that IGF-1 increases AKT phosphorylation and enhance eNOS activation induced by IGL-1® alone. This in turn induces NO generation, which reduces vascular resistance following reperfusion. This is in line with several reported studies demonstrating that a low concentration of eNOS-derived NO maximizes blood perfusion, promotes cell survival and protects the liver against IRI[39]. In contrast, the sustained presence of iNOS-derived NO might become detrimental by increasing toxic reactive oxygen species, thus leading to liver injury[39-41]. In our experimental model, the benefits of NO were not associated with iNOS activation (data not show). Moreover, other studies demonstrate that while increasing e-NOS, IGF-1 inhibits inducible NO[19,42].

It is clear that fatty accumulation resulting from steatosis induces ultra-structural and biochemical changes in liver mitochondria[43,44], which may render these organelles intrinsically more susceptible to IRI injury. Given that mitochondria are the main sites of ROS production in IRI, the increased oxidative stress observed in steatotic livers after cold IRI could be attributed to mitochondrial damage. It is well known that IGF-1 prevents mitochondrial damage and oxidative stress following IRI[17,33,34]. Our results indicate that the addition of IGF-1 to IGL-1® preservation solution increases liver mitochondria protection, and prevents ROS generation associated with reperfusion injury.

Vairetti *et al*[45] using the same "*ex vivo*" experimental model, have shown that TNF-α is released when fatty livers are subjected to cold ischemia reperfusion. In our conditions, TNF-α release was also evidenced in response to cold ischemia-reperfusion insult.

IGF-1 supplementation prevented the release of TNF-α[46], which has a pivotal role in the progression of liver reperfusion damage. This finding is consistent with the accumulating evidence of crosstalk between IGF-1 and TNF-α during ischemia reperfusion injury[47]. NO generated by IGF-1 could decrease TNF-α release, as occurs in liver ischemic preconditioning, in which the induced hepatoprotection is also mediated by the inhibitory action of NO on TNF-α release through eNOS activation[48].

In addition, several intracellular signalling pathways, including the extracellular signal-regulated kinases 1/2 and P38 mitogen-activated protein kinase, are activated during hypothermia[49-52], In these conditions, a mutual activation effect between TNF-α and MAPKs may also occur thus exacerbating the liver damage[53]. Our results indicate that cold storage in IGL-1® solution alone activates P38 and ERK 1/2, and that IGF-1 addition prevented this activation, which is consistent with a TNF-α reduction that leads to an improvement in liver injury and function. This is in line with previous reports that the addition of TF to UW improved endothelial cell preservation by reducing ERK 1/2 and P38 activation[22].

In conclusion, we have demonstrated that IGF-1 addition to IGL-1® solution protects fatty liver against cold IRI. The beneficial action of IGF-1 appears to be mediated by AKT activation and NO generation, with concomitant prevention of pro-inflammatory cytokines, such as TNF-α. However, further studies will be required to examine the underlying mechanisms. This may increase the use of steatotic livers, which partially compensates the shortage of organs for transplant.

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COMMENTS

Background

Static preservation solution is crucial for graft viability; especially when steatosis is present. Serum-free preservation solutions deprive the donor organ of essential trophic factors such as insulin like growth factor-1 (IGF-1) which is associated with deleterious effects including cell cycle arrest and death. The addition of IGF-1 to University of Wisconsin (UW) solution improves the capacity of this preservation solution for protecting liver grafts subjected to a prolonged ischemic period. Serum-free institut georges lopez-1 (IGL-1) solution has been proposed as an effective alternative to UW for steatotic liver preservation.

Research frontiers

In this study, the authors focused on the importance of IGF-1 as a biological additive to IGL-1 preservation solution. The induction of nitric oxide (NO) synthesis by the modified IGL-1 solution improves microcirculatory disorders in fatty livers. IGL-1 supplementation with IGF-1 increases graft protection against ischemia reperfusion injury through the activation of protein kinase AKT and a concomitant reduction in tumour necrosis factor (TNF) release and P38/ERK 1/2 MAPKs activation which play an important cryoprotective role in liver graft preservation.

Innovations and breakthroughs

The enrichment of IGL-1 solution with IGF-1 increases NO generation through AKT activation and prevents pro-inflammatory cytokine release such as TNF-α during steatotic liver graft reperfusion.

Applications

The use of modified IGL-1 solutions should be a useful strategy for increasing steatotic liver graft preservation.

Peer review

This manuscript describes the beneficial effects of IGF-1 when added to IGL-1 solution in the preservation of (fatty) liver.

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ORIGINAL ARTICLE

Redefining the properties of an osmotic agent in an intestinal-specific preservation solution

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Abstract

AIM: To investigate the effects of dextrans of various molecular weights (Mw) during a 12 h cold storage time-course on energetics, histology and mucosal infiltration of fluorescein isothiocyanate (FITC)-dextran.

METHODS: Rodent intestines were isolated and received a standard University of Wisconsin vascular flush followed by intraluminal administration of a nutrient-rich preservation solution containing dextrans of varying Mw: Group D1, 73 kdal; Group D2, 276 kdal; Group D3, 534 kdal; Group D4, 1185 kdal; Group D5, 2400 kdal.

RESULTS: Using FITC-labeled dextrans, fluorescent micrographs demonstrated varying degrees of mucosal infiltration; lower Mw (groups D1-D3: 73-534 kdal) dextrans penetrated the mucosa as early as 2 h, whereas the largest dextran (D5: 2400 kdal) remained captive within the lumen and exhibited no permeability even after 12 h. After 12 h, median injury grades ranged from 6.5 to 7.5 in groups D1-D4 (73-1185 kdal) representing injury of the regenerative cryptal regions and submucosa; this was in contrast to group D5 (2400 kdal) which exhibited villus denudation (with intact crypts) corresponding to a median injury grade of 4 ($P < 0.05$). Analysis of tissue energetics reflected a strong positive correlation between Mw and adenosine triphosphate ($r^2 = 0.809$), total adenylates ($r^2 = 0.865$) and energy charge ($r^2 = 0.667$).

CONCLUSION: Our data indicate that dextrans of Mw > 2400 kdal act as true impermeant agents during 12 h ischemic storage when incorporated into an intraluminal preservation solution.

Key words: Intraluminal preservation solution; Intestinal-specific; Osmotic impermeant; Organ preservation; Cold storage

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INTRODUCTION

Small bowel transplantation (SBT) has become an essential treatment for patients with irreversible intestinal failure who do not succeed on parenteral supplementation[1]. The global frequency and success rates of such procedures have seen steady increases over the last decade[1]. While only 11 intestinal transplants were performed in 1990, 140 cases were reported in 2003; currently approximate 200 are performed annually across the world[1]. For those individuals receiving antibody-based induction therapy and tacrolimus-based maintenance immunosuppression, one year post-transplant survival rates are comparable to those of liver (> 80%)[1]. The majority of individuals do not receive these therapies, hence 5-year graft survival rates for SBT remain low (31%-69%; 48% weighted average for 2005 data) compared to other commonly transplanted organs[2].

Among the obstacles to successful SBT is the extreme susceptibility of the mucosal epithelium to even brief periods of ischemia[3]. For this reason, the ability to successfully preserve graft viability during the period of ischemic cold storage is critical. Preventing hypothermia-induced cellular swelling is a basic principle of successful organ preservation[4]. One major advancement in preservation solution technology and design was the development of the "gold standard" preservation solution, the University of Wisconsin (UW) solution. Improvements in organ quality and safe cold ischemic times can be largely attributed to the control of cellular edema. This was accomplished by including cell-impermeant molecules lactobionate, raffinose, and hydroxyethylstarch (HES)[5]. With respect to the intestine, studies have shown that net fluid shifts contributing to mucosal injury can originate from the vasculature or lumen[6,7]. Since standard intestinal procurement involves a common intra-aortic flush of all abdominal organs, current preservation strategies do not address fluid shifts of a luminal origin.

Throughout the last several years, our laboratory has developed a novel nutrient-rich preservation solution (containing a large amino acid component) tailored to the specific metabolic requirements of the small intestine; termed AA solution. Numerous *in vitro* and *in vivo* models have documented superior maintenance of energetics, a reduction in oxidative stress, and a preservation of mucosal morphology and barrier function following intraluminal administration of the nutrient-rich solution[8-10]. We have demonstrated the benefits of using a high molecular weight (Mw) HES (2200 kdal) as an impermeant molecule in our novel solution[11,12], with no direct evidence that the starch molecule does not penetrate the mucosal barrier during ischemia. Recent data from our lab has demonstrated that dextran (Mw = 70 kdal) is not osmotically active when delivered as part of an intraluminal preservation solution; direct visualization of a fluorescently-labeled dextran-70 clearly showed the rapid migration of the dextran into the mucosa. This has raised the question as to the exact Mw characteristic of an effective impermeant agent in the realm of intestinal ischemia as it relates to a novel strategy of intraluminal preservation.

Although the UW solution contains HES on the basis of preventing interstitial edema incurred during cold ischemia[4], there exists controversy over its effectiveness for static organ storage. Some studies report that HES may be omitted from the UW solution without detrimental effects on overall graft quality[13-15], while others suggest a protective role of HES in stored tissues[16-20]. Because these previous studies deal solely with an intravascular delivery of UW solution, they do not provide information about the role of colloids in preservation solutions administered intraluminally for intestinal grafts. We suspect that during cold storage, alterations in the permeability characteristics of the mucosa will result in the infiltration of relatively large osmotic agents, thereby failing to effectively control tissue edema and mucosal viability. We hypothesized that there is a critical Mw for an effective osmotic agent to be used in an intraluminal preservation solution during cold ischemic storage.

MATERIALS AND METHODS

Summary of experimental design

Briefly, small intestines from rats were flushed intravascularly with UW solution, isolated and flushed intraluminally with a nutrient-rich preservation solution containing 5% dextran of varying Mw (73, 276, 534, 1185, 2200 kdal). Fluorescein isothiocyanate (FITC)-labeled dextrans to a final fluorescence of 10×10^6 fluorescence units/mL were incorporated into the solutions to aid in direct visualization of dextran infiltration. Intestines were stored at 4℃ and samples were taken over a 12 h time-course for subsequent analysis.

Surgical procedure and intestinal procurement

Male Sprague-Dawley rats (200-250 g) were obtained from the University of Alberta and used as organ donors. All experiments were conducted in accordance with Canadian Council on Animal Care policies. Animals were fasted overnight and provided water *ad libitum*. Rats were induced with pentobarbital (65 mg/250 g; IP), followed by inhalational isoflurane (0.5%-2%) to maintain anesthesia. Following a midline laparotomy, the aorta was exposed infrarenally and at the celiac trunk. The supraceliac aorta was clamped and 2-4 mL modified UW solution was administered *via* the infrarenal aorta. The vena cava was transected to facilitate the outflow of blood and perfusate. The entire jejunum and ileum was subsequently harvested. A nutrient-rich preservation solution (AA solution), developed in our lab, was used to flush and clear the lumen of its contents (40 mL; approximate 2.0 mL/g). Sixteen centimeter-long sections of ileum were measured out. The sections were filled at the proximal end with 4-5 mL nutrient-rich preservation solution containing 5% dextran of varying Mw (fluorescence was standardized to 10×10^6 fluorescence units/mL and each end ligated with 3-0 silk). The preservation solution contained (values in brackets are mmol/L): Glutamine (35), Lactobionate (20), Glutamate (20), Aspartate (20), Glucose (20), BES [N,N-Bis(2-hydroxyethyl)taurine] (15), Arginine (10), Glycine

Table 1 Characteristics of high-purity dextrans

Group	Mp	Mw	Mn	PDI	IV
D1	62900	72700	50700	1.43	0.263
D2	238000	275900	204600	1.35	0.463
D3	490000	534000	371000	1.43	0.633
D4	1050000	1185000	705000	1.67	0.862
D5	2000000	2400000	1600000	1.50	0.803

Mp: Peak average molecular weight; Mw: Weight average molecular weight; Mn: Number average molecular weight; PDI: Polydispersity index (PDI = Mw/Mn); IV: Intrinsic viscosity (dL/g).

(10), Asparagine (10), Threonine (10), Lysine (10), Valine (10), Serine (10), Methionine (5), Leucine (5), Isoleucine (5), Histidine (5), Ornithine (5), Proline (5), Adenosine (5), Cysteine (5), β-Hydroxybutyrate (3), Tyrosine (1), Tryptophan (1), Trolox (1), 3-Aminobenzamide (1), Allopurinol (1); plus the inclusion of 5% Dextran. The pH of each solution was adjusted to 7.40 with sodium hydroxide; measured osmolarity was 320 mOsm.

Group designations were as described in Table 1; briefly the dextran Mw for the 5 groups (D1-D5) were: 73 (D1); 276 (D2); 534 (D3); 1185 (D4); 2400 (D5) kdal.

Tissues were stored on ice at 4℃ in standard AA solution and sampled at 2, 4, and 12 h; at each time-point a 4 cm length of intestine was sampled; half was used for histology and half for metabolite analysis. To arrest metabolic activity, samples were snap frozen in liquid nitrogen, and stored at -65℃ until processed.

FITC-labeling of dextrans

Dextrans used in this study were obtained from American Polymer Standards Corporation, Ohio, US; these dextrans were of the highest purity available and characteristics are provided in Table 1. Briefly, peak average molecular weight (Mp), weight average molecular weight (Mw), number average molecular weight (Mn), and polydispersity index (PDI) describe molecular distribution curves. The key parameter defining dextran purity is the PDI with a value of 1.0 representing absolute purity. PDI values of less than 3 represent high purity fractions; hence values in the current study of 1.35 to 1.67 are of extremely pure dextran fractions. The procedure used to FITC-label the dextrans from the above solutions was based on the original procedure used by De Belder and Granath[21]. The dextran of interest together with fluorescein 5-isothiocyanate (FITC) were dissolved in dimethyl sulphoxide, pyridine and dibutyltin dilaurate and heated to 95℃ for 2 h. The mixture was then put on ice and the dextran was precipitated out with cold 100% ethanol, filtered and dried in an oven at 40℃. Labeling efficiency was 0.015-0.020; this corresponds to 1 fluorescein molecule per 50-67 glucose units. FITC-labeled dextran accounted for 8.5% ± 0.6% of total dextran in each solution; fluorescence was standardized. Fluorescence for FITC-dextrans was evaluated at an excitation wavelength = 485 nm/emission wavelength = 535 nm.

Stability of the FITC label

When labeling fluorescent moieties to target molecules, one of the key concerns that must be considered for subsequent experimentation to be valid is that the fluorescent label must remain bound to the target and does not spontaneously detach over time when in aqueous solution. In this study, a dilute solution (approximate 0.1%) of each dextran was incubated at 4℃ for 24 h. Following the incubation period, FITC-dextrans were precipitated with cold 100% ethanol, centrifuged, and the supernatant (containing free FITC) was assessed for fluorescence. When solutions were incubated at 4℃, there were no statistically significant decreases in FITC-bound dextrans; this indicated a stable conjugation for all high-purity dextrans used in this study.

Sample preparation and metabolite assay

Frozen small bowel samples were weighed and then extracted 1:5 weight/volume in perchloric acid containing 1 mmol/L ethylenediamine tetra-acetic acid. The precipitated protein was removed by centrifugation (20 min at 20000 × *g*). Acid extracts were neutralized by the addition of 3 mol/L KOH/0.4 mol/L Tris/0.3 mol/L KCl and then recentrifuged (20 min at 14000 × *g*). Aliquots of neutralized extracts were immediately processed *via* standard enzyme-linked metabolite assays[22]. Spectrophotometric analysis was then performed to measure the absorbance of NADH at 340 nm, providing quantification of adenosine triphosphate (ATP), total adenylates [ATP + adenosine diphosphate (ADP) + adenosine monophosphate (AMP)] and energy charge [(ATP + 0.5 ADP)/total adenylates] and malondialdehyde[22,23]. Values are reported as μmol per gram protein. Protein was measured according to the method of Lowry *et al*[24].

Histology

Bowel samples were fixed in alcohol formalin solution, processed to paraffin wax, embedded, and sections cut at 5 μm. Sections were dewaxed then mounted with Prolong Gold anti-fade reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) and sealed with nail polish. Fluorescent microscopy was used to view FITC and DAPI fluorescence in tissues; wavelengths were Ex = 485/Em = 535 and Ex = 360/Em = 460, respectively. Fluorescent images were digitally captured using AxioVision software. All photos shown reflect representative findings. A second set of sections were stained with hematoxylin and eosin and graded according to a modified Park's classification for intestinal injury[25] as follows.

Grade 0: Normal mucosa; grade 1: Subepithelial space at villus tip; grade 2: Moderate subepithelial space; grade 3: Epithelial lifting along villus sides; grade 4: Denuded villi; grade 5: Loss of villus tissue; grade 6: Crypt layer injury; grade 7: Transmucosal injury; and grade 8: Transmural injury.

Statistical analysis

Metabolite data were reported as mean ± SE for each

Table 2 Intestinal morphology following cold ischemic storage

Time (h)	Group	Grade	Median	Significance	Event
2	D1	0, 2, 6, 7	4		Denuded villi
	D2	3, 4, 6, 6	5		Loss of villi
	D3	1, 2, 2, 3	2	D3, D4, D5 *vs* D1, D2, $P = 0.06$	Moderate clefting
	D4	1, 2, 3, 5	2.5		Moderate-extensive clefting
	D5	1, 2, 3, 4	2.5		Moderate-extensive clefting
12	D1	0, 6, 7, 8	6.5		Injury to crypts and submucosa
	D2	2, 6, 7, 7	6.5		Injury to crypts and submucosa
	D3	3, 7, 8, 8	7.5		Injury to crypts, submucosa and muscularis
	D4	3, 6, 8, 8	7		Injury to crypts and submucosa
	D5	0, 4, 4, 5	4	D5 *vs* D1-D4, $P < 0.05$	Clefting and denuded villi

A modified Park's classification for intestinal injury was used to assess mucosal morphology as follows: Grade 0: Normal mucosa; Grade 1: Subepithelial space at villus tip; Grade 2: Moderate subepithelial space; Grade 3: Epithelial lifting along villus sides; Grade 4: Denuded villi; Grade 5: Loss of villus tissue; Grade 6: Crypt layer injury; Grade 7: Transmucosal injury; Grade 8: Transmural injury.

group. Statistical differences between groups were determined using analysis of variance, followed by Student-Newman-Keuls'. Analysis of relationships between Mw and ATP, total adenylates, or energy charge was performed with a linear regression analysis for parametric data. Differences in histology grades were assessed by a non-parametric Kruskal-Wallis test.

RESULTS

Infiltration of FITC-labeled dextrans during cold ischemic storage

The lower Mw dextrans tested (D1, D2, D3) consistently demonstrated infiltration of the fluorescent label at all time-points. At the earlier time-points, 2 and 4 h, fluorescence intensity was greatest in the epithelium or in sloughed epithelial cells. After 12 h storage, there was significant infiltration into the cryptal regions, the lamina propria, as well as the vascular epithelium. Mucosal injury was greatest in these groups (D1, D2, D3), ranging from development of subepithelial clefts at 2 h to complete denudation at the later time-points, leaving the underlying tissue vulnerable to further dextran penetration.

Interestingly, the intermediate Mw dextran, D4, exhibited no extensive penetration of label into the epithelial layer and largely remained within the layer of mucous coating the surface of the villi. Several goblet cells and the apices of some villi did have a minor amount of fluorescence. However, by 4 h, there was considerable infiltration of the dextran label throughout the underlying tissue. Limited mucosal injury was apparent even after 12 h storage, at which time epithelial clefting had developed. Treatment with the highest Mw tested, D5, resulted in no observable infiltration into the epithelium or lamina propria; this was evident at all time-points (Figure 1).

Evidence of mucosal injury

Histologic injury was evident early on during cold storage, particularly for the lower Mw dextrans after only 2 h. Most notably, in groups D1 and D2, 50% of specimens exhibited a significant degree of crypt infarction, compared to no evidence of crypt infarction in groups D3, D4, and D5 ($P = 0.06$).

By 12 h, 75% (12/16) of specimens in groups D1-D4 had an injury grade of 6 or greater (damage to the crypts, mucosa, or transmural injury). Conversely, 75% of specimens in Group D5 exhibited clefting where the epithelium had lifted off the underlying lamina propria (grade 3) and at times had dissociated from the villus proper (grade 4) ($P < 0.05$). A summary of injury grading and median grades are presented in Table 2. Representative fields of injury are presented in Figure 2 micrographs.

ATP, total adenylate, energy charge levels

Following 12 h cold storage, ATP levels ranged from 5.3 to 9.6 μmol/g, with D1 and D2 having the lowest levels (5.3-5.6 μmol/g). Intermediate Mw groups, D3 and D4, exhibited intermediate ATP levels, both significantly different from D1 and D2 ($P < 0.05$). ATP progressively increased to a maximum of 9.6 μmol/g in D5; this group had significantly greater levels than all other groups ($P < 0.05$). Levels of total adenylates (ATP + ADP + AMP) closely resembled those of ATP. Levels in groups D2 and D3 were significantly higher than in D1 and D2, $P < 0.05$. Similarly, D5 level was significantly greater than all groups, $P < 0.05$. Atkinson described another useful measure of tissue energetics, "energy charge" as: EC = (ATP + ADP/2)/total adenylates[26]. In illustrating the significance of this measurement, Pegg used the analogy of a battery in describing the energy charge as being a measure of the "charged up" state of the adenylate pool[27]. As ATP levels decline relative to ADP and AMP levels, energy charge also drops, indicating that less of the total adenylate pool exists in a form that is immediately available for cellular work. Energy charge ratios showed similar trends to those of ATP and total adenylates. Values increased as Mw of the dextran increased, starting at 0.45 in D1 and reaching 0.62 in D5; D5 value was significantly different than all values in groups D1-D4 ($P < 0.05$) (Figure 3).

Correlation between energetics and dextran Mw

With respect to dextran Mw, there was a strong positive and statistically significant correlation with ATP ($r^2 = 0.809$); total adenylates ($r^2 = 0.865$); and energy charge

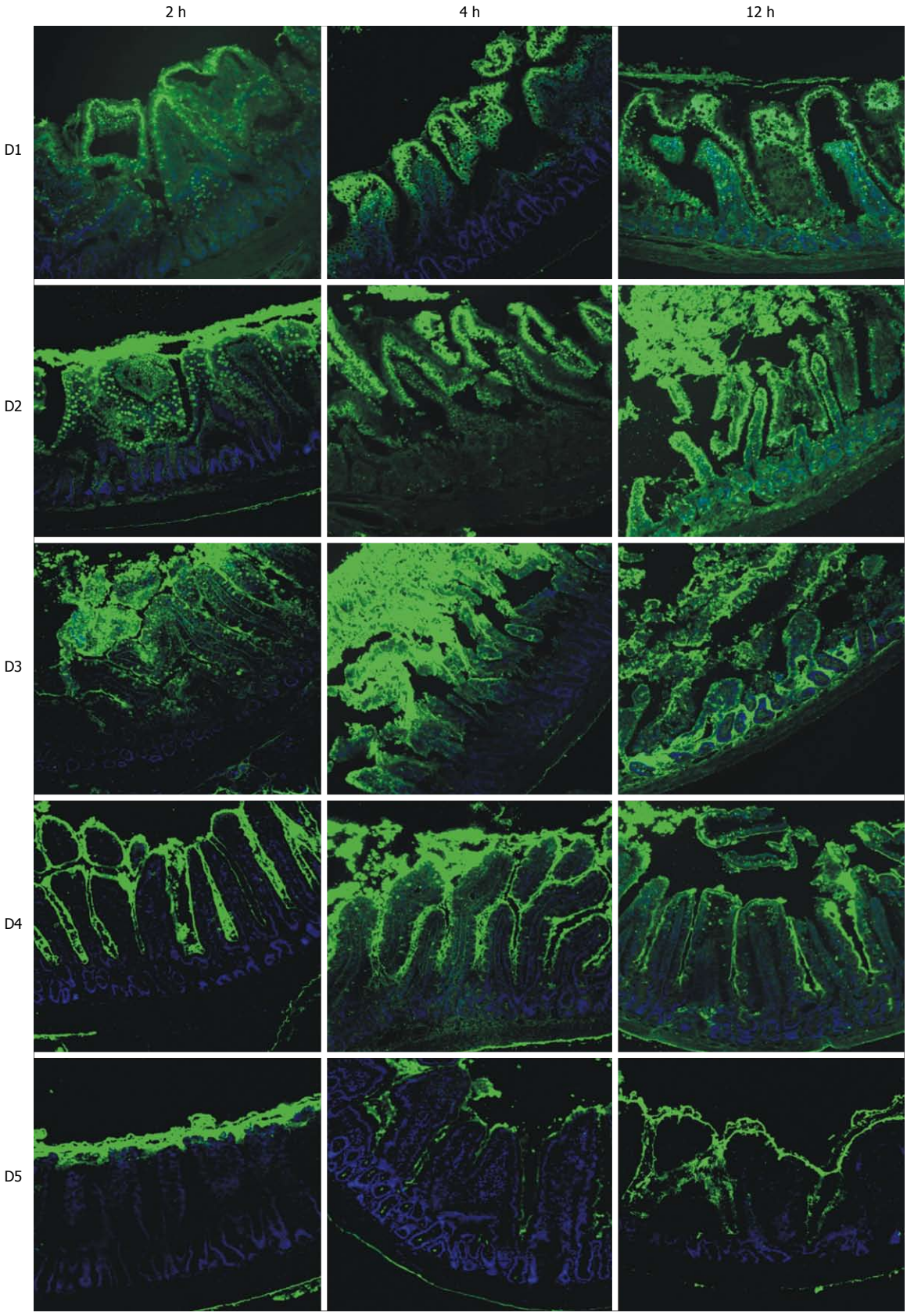

Figure 1 Infiltration of fluorescein isothiocyanate-labeled dextrans during cold ischemic storage. All magnifications (× 10 objective) are of representative fluorescent intensities and mucosal injury. All photos have a standardized exposure time of 250 ms for the fluorescein isothiocyanate channel; exposure times for 4',6-diamidino-2-phenylindole, a common nuclear stain, are 2-20 ms and are included for contrast.

(r^2 = 0.667). Overall, group D5 was the most effective in preserving the three parameters of tissue energetics over the 12 h period of cold ischemic storage, while lower Mw groups exhibited poor conservation of energetics. Supe-

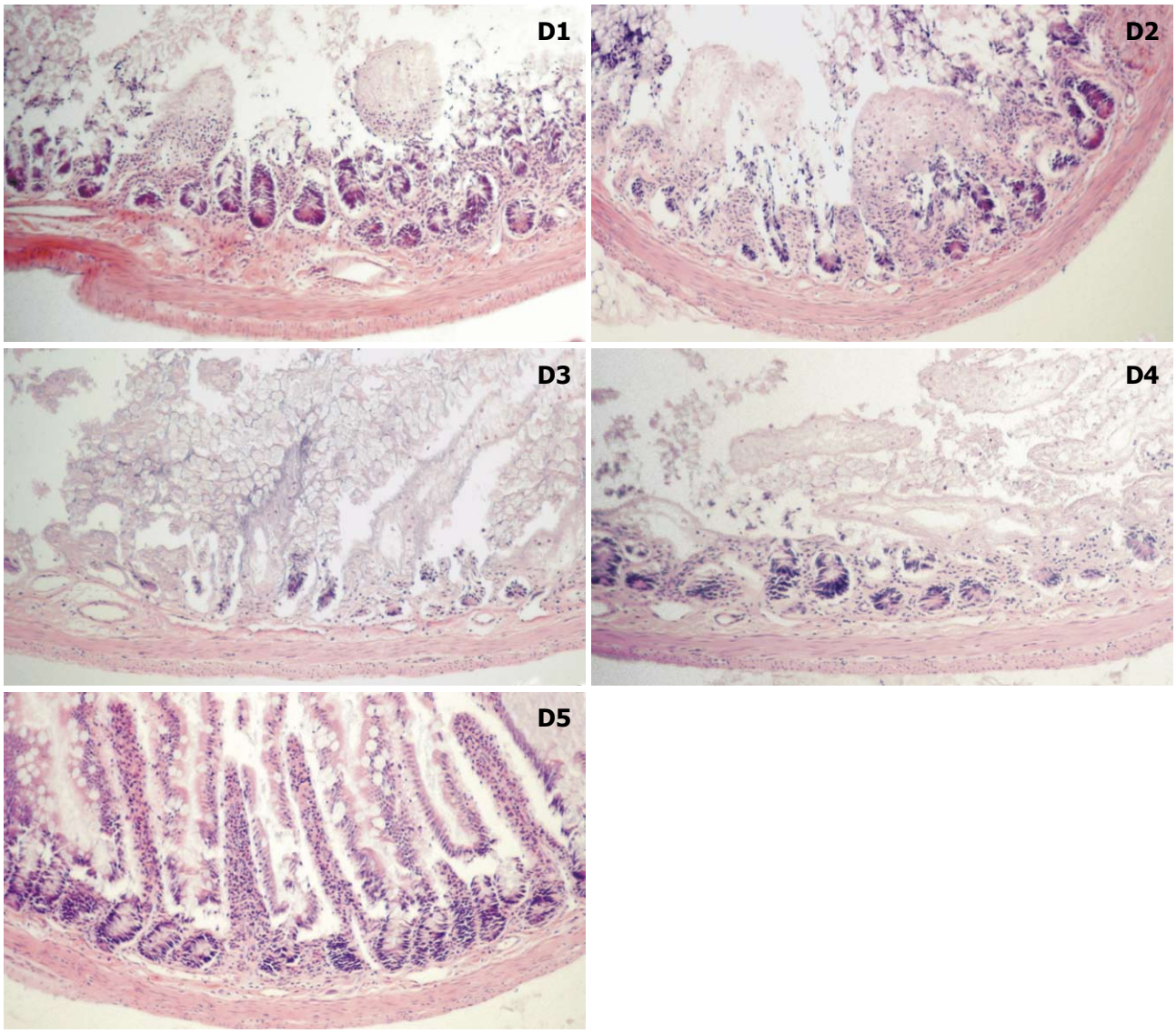

Figure 2 Histology after 12 h cold ischemic storage. Photos are of representative median grades of histologic injury according to a modified Park's classification of intestinal injury[25]. Magnification with 10 × objective. HE staining, 5 μm sections. D1: Grade 6.5; D2: Grade 6.5; D3: Grade 7.5; D4: Grade 7; D5: Grade 4.

rior maintenance of energetic parameters supported the preservation of mucosal viability as determined by direct histologic observation of mucosal morphology as well as penetration of the FITC-labeled dextrans (Figure 4).

Oxidative stress

No significant differences were detected among groups D1-D5 with respect to the parameter of oxidative stress (malondialdehyde) after 12 h storage; values varied between 236 and 274 nmol/g protein in all 5 experimental groups (data not shown).

DISCUSSION

The major clinical application of dextrans, in addition to other starches and albumin, has been for their plasma volume expansion properties. These molecules exert their osmotic effects by binding water within the vasculature and creating an osmotic force to extract water from edematous tissues. The Mw of a molecule required to remain captive within the vasculature is very low as demonstrated by the effectiveness of an intravascular flush during organ procurement with the UW solution (osmotic agents, lactobionate and raffinose; Mw = 358 and 504, respectively). With respect to osmotic support and the application of an intraluminal preservation solution, the Mw permeability limit of the epithelial barrier during ischemic storage has not been clearly defined.

In the realm of organ preservation, osmotic agents are required to counteract the metabolic events that result in the influx of water into the cells/tissue. Under normal circumstances, cells have an extracellular environment rich in Na^+ and low in K^+[5]. The Na^+/K^+ transporter maintains the respective gradients of these ions, relying on the use of ATP as its energy source. Essentially, this pump makes Na^+ an impermeant outside of the cell, creating an osmotic force that counteracts the osmotic pressure exerted by proteins and impermeable anions in the cell interior. Together, these intracellular proteins and impermeable ions require an osmotic force of 110-140 mOsm/kg to

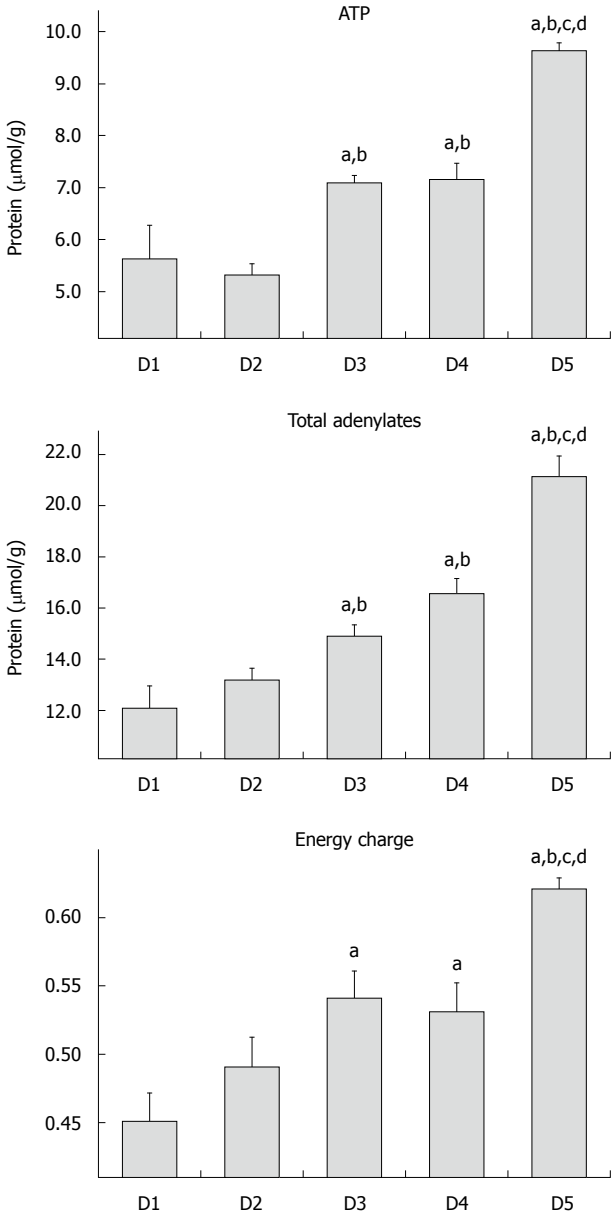

Figure 3 Parameters of energy metabolism during cold ischemic storage. Adenosine triphosphate (ATP), total adenylates and energy charge levels are presented. $^{a,b,c,d}P < 0.05$, significantly different *vs* D1, D2, D3, D4, respectively.

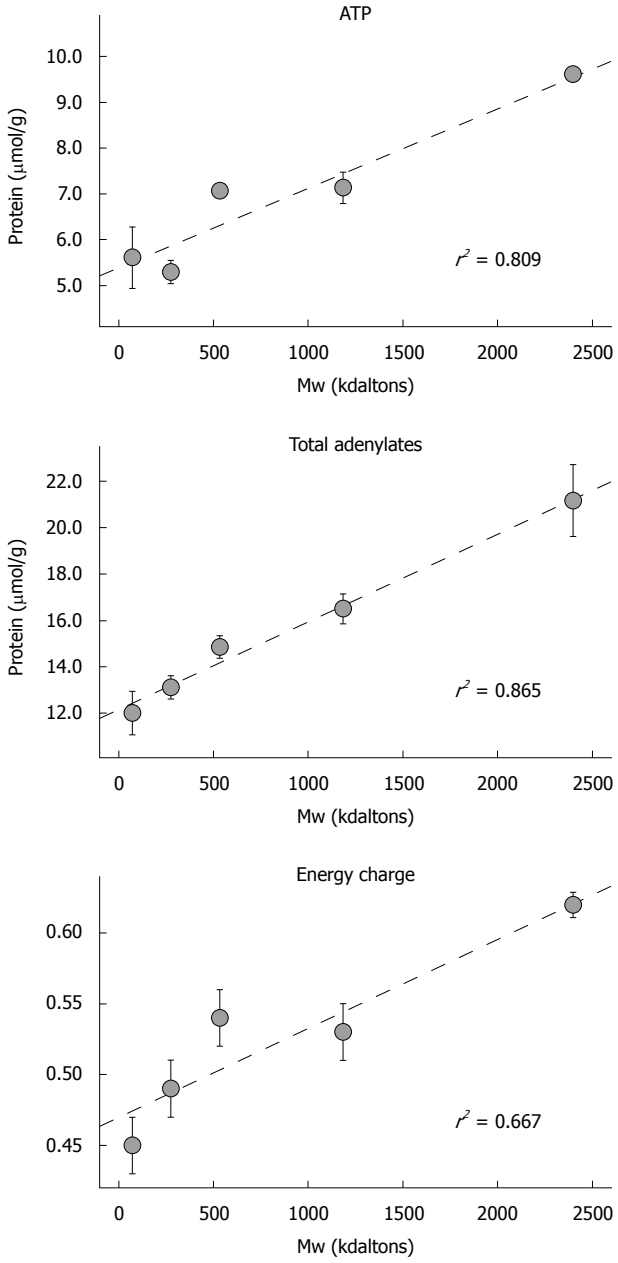

Figure 4 Correlation between dextran size and energy metabolism. Linear regression analysis was performed; correlation coeffients (r^2) are presented. ATP: Adenosine triphosphate; Mw: Molecular weight.

offset fluid flow into the cell[5]. In ischemic tissue under cold storage, the above situation is quite different. Hypothermic conditions coupled with aerobic metabolism collectively work to inhibit Na^+/K^+ ATPase activity, decreasing the cell membrane potential. As a result, Na^+ and Cl^- flow down their concentration gradients into the cell, and water follows, causing the cell to swell; cells eventually become damaged and may lyse[5,19]. Cell impermeant molecules are critically important in regulating fluid movement into the cell; there is a fundamental requirement of 110-140 mmol/L of osmotic agents in order to counteract cell swelling[5].

When utilizing an intraluminal preservation solution, inadequate osmotic support will culminate in direct injury to the epithelium. This leads to the compromise of cellular integrity and consumption of essential energy reserves in an attempt to repair damaged tissue. Depletion of cellular energy reserves (ATP and total adenylates) results in an inability of the mucosal epithelium to preserve its barrier function, compromising epithelial integrity during organ storage[20,28]. The barrier consists of numerous tight junctions located toward the apical surface of intestinal epithelial cells[8]. A number of proteins form the tight junctions, which are active, energy-consuming structures[8]. The transepithelial flow of both macromolecules and enteric bacteria are thought to be caused by tight junction dilation[3], and the consequences are twofold: (1) an increased likelihood of sepsis and (2) a need to consider the permeability of molecules used for osmotic support during preservation. Sepsis may occur due to bacterial translocation[29,30], and continues to account for the majority

of deaths after intestinal transplantation (46.0% between April 1985 and May 2003)[1]. Increased permeability of the intestine during ischemic conditions should influence the critical Mw limit for molecules added to the intraluminal preservation solution for impermeant support. Inclusion of the appropriate agent capable of acting as a true osmotic will improve the quality of the intestinal graft and ultimately influence success of the transplant.

Hydrostatic and impermeant forces regulate the movement of fluids between intracellular and extracellular compartments. Hydrostatic forces have a predominant role in continuous perfusion methods; whereas, impermeant forces are the sole contributors governing net fluid flux in the setting of static cold storage, the current standard for most intra-abdominal organs. Shifts in net fluid flow during cold storage are proposed to originate from the intestinal vasculature and/or lumen[6,7]. In the current study, fluid shifts of vascular origin were controlled by delivering a standard intra-aortic vascular flush with UW solution to all treatment groups, as would happen in the clinical situation. Keeping such fluid shifts constant was essential, allowing us to attribute any inter-group differences of luminal permeability to the specific dextran size of interest. Based on the data presented in the current communication, there was a definitive inverse relationship between dextran Mw and its ability to cross the epithelial barrier during cold storage. Several relatively large Mw dextrans (73-534 kdal) exhibited a definitive pattern of penetration of the mucosal layer within 2 h cold storage, eventually infiltrating the submucosa and muscularis propria after 12 h. The only effective dextran fraction tested that exhibited impermeant characteristics by remaining captive within the lumen even after 12 h cold ischemia was one of Mw = 2400 kdal. This study is the first to establish the critical permeability limit of the mucosal layer during intestinal preservation and to delineate the requisite Mw of a true osmotic impermeant in the lumen.

The relationship between Mw (size) and permeability determined in this study was somewhat expected based on the results from various animal and human intestinal permeability studies[15,17,18]. An inverse relationship between Mw and mucosal-to-serosal permeability has been established previously for rodents[17]. In a comprehensive report addressing comparative intestinal permeability in pig, rat, and human models, Nejdfors *et al*[15] found an inverse relationship between the Mw of marker molecules and their mucosal permeability across all species, irrespective of location in the gastrointestinal tract. The power of such a study comes from the fact that identical standardized methods of evaluating molecular permeability and a wide range of Mw were used for each species; this included ^{14}C-mannitol, FITC-dextrans 4.4/70 kdal and several proteinaceous macromolecules (α-lactalbumin, 65 kdal and ovalbumin, 45 kdal).

In an animal study assessing the uptake of FITC-dextran (Mw 70 kdal) by the small intestinal epithelium, Ekström *et al*[29] found that during neonatal development (< 30-d old) there were negligible amounts of FITC-dextran in the serum with no enterocyte infiltration. One should note that this experiment was conducted in healthy fully oxygenated tissues without any prior ischemic insult. Based on such reports, our nutrient-rich solution has in the past contained a dextran fraction with similar Mw for impermeant support[8,30,31]. There is no reason to suspect that a macromolecule which is impermeant in the lumen of a healthy bowel will do so during ischemic conditions, even at reduced temperatures. As the number of tight junctions and the magnitude of the dilatations increase, the potential for infiltration and translocation of large macromolecules and eventually bacteria also increases. As a consequence of these impermeant characteristics, the lack of osmotic support contributes directly to tissue edema and its negative sequelae. In tissues lacking sufficient impermeant support during hypothermic storage, edema becomes a major concern.

In the current study, parameters of cellular energetics (ATP, total adenylates and energy charge) consistently reflected strong and significant positive correlations with respect to dextran Mw. Although the value of ATP to the cell is obvious, higher Total Adenylate levels reflect reduced rates of purine catabolism, potentially leading to the accumulation of hypoxanthine and the production of uric acid and superoxide (a highly reactive oxygen free radical)[32]. A second consequence is that greater amounts of purines are available for ATP regeneration (once carbon exits the total adenylate pool, reconversion is not enzymatically possible in this pathway). These biochemical parameters of tissue integrity all indicate that the 2400 kdal dextran was maximally effective in protecting the mucosa from ischemic insult during cold storage.

In conclusion, the permeability characteristics of the rat small bowel during cold ischemia are very different than those of intestinal tissue that is not limited with respect to oxygen and nutrients. In order for a molecule to behave as a true osmotic impermeant, the Mw must be much greater than that established for intravascular preservation solutions. From the data presented in this study, the size of dextran showing the lowest degree of epithelial or mucosal infiltration, superior morphology and minimal disruption to cellular energetics was 2400 kdal. These findings clearly delineate the critical Mw limit that must be addressed in the development of an effective intraluminal preservation solution.

ACKNOWLEDGMENTS

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COMMENTS

Background

An effective osmotic agent is one of the fundamental requirements of organ preservation for transplantation. Commonly used preservation solutions that are typically administered *via* an intravascular route utilize low molecular weight (Mw) impermeant compounds to maintain tissue water balance. Our laboratory has developed a novel strategy for intestinal preservation involving the intraluminal delivery of a nutrient-rich preservation solution; however, an appropriate impermeant molecule has not been clearly defined.

Research frontiers

Although organ preservation methods have existed for decades, luminal administration of a preservation solution tailored to the metabolic requirements of the small bowel is a novel concept.

Innovations and breakthroughs

This study reveals for the first time that a large Mw impermeant molecule is a fundamental requirement of an intraluminal preservation solution during cold, static storage of the small intestine. Interestingly, only the highest Mw dextran (2400 kdal) remained within the intestinal lumen throughout the entire 12 h period of cold storage, resulting in superior graft energy status and tissue morphology.

Applications

The current study focused on defining a suitable osmotic agent for use in our intraluminal preservation solution, a key factor affecting intestinal viability during organ storage. As a back-table luminal flush that does not interfere with the clinical vascular preservation method, our AA solution has the potential to revolutionize intestinal preservation, and therefore improve patient outcomes following transplantation.

Peer review

The manuscript submitted by Schlachter *et al* represents a methodical and well written study investigating the protective effects of different Mw dextrans in a novel intestinal preservation solution. The findings of the study are potentially important and widely applicable to the intestinal transplantation field.

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BRIEF ARTICLE

Role of simple biomarkers in predicting fibrosis progression in HCV infection

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Abstract

AIM: To examine the accuracy of the aspartate aminotransferase (AST)/Platelet Ratio Index (APRI) and FIB-4, in predicting longitudinal changes in liver histology in hepatitis C virus (HCV) patients.

METHODS: Patients that underwent repeat liver biopsies at least 1 year apart from 1999 to 2007 were identified. Liver fibrosis was staged on needle core biopsies evaluated by a single expert liver pathologist. Only laboratory values within 3 mo of the liver biopsies were used.

RESULTS: Thirty-six patients met the inclusion criteria with 50% stage 1 on initial biopsy, 25% stage 2, and 22% stage 3. Nineteen of 36 (53%) had progression of fibrosis on repeat biopsies, while 16 (44%) showed no change in stage, and one (3%) showed improvement. Patients that showed progression of fibrosis had significantly higher alanine aminotransferase and aspartate aminotransferase levels than the group that did not show progression. A significant correlation was seen between change in stage of fibrosis and change in APRI (r^2 = 0.39, P = 0.00001) and a change in FIB-4 (r^2 = 0.31, P = 0.00004). A change in APRI (ΔAPRI) of 0.18 had 80% positive predictive value (PPV) and 67% negative predictive value (NPV) for progression of fibrosis. A change in FIB-4 (ΔFIB-4) of 0.39 had 75% PPV and 75% NPV for predicting progression of fibrosis.

CONCLUSION: ΔAPRI and ΔFIB-4 parallel changes in fibrosis progression, and could be useful tools for clinicians in following patients with active chronic HCV infection.

Key words: Hepatitis C; Liver fibrosis; Liver biopsy; Biomarkers

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INTRODUCTION

Hepatitis C virus (HCV) infection is one of the most common causes of chronic liver disease, and affects ap-

proximately 3% of the world's population[1]. Current treatment decisions in chronic HCV are guided by the histopathological findings on liver biopsy. Although it is still considered the gold standard for assessment of fibrosis in chronic hepatitis C, liver biopsy has limitations. It is an expensive and invasive procedure with complications that range from pain (0.06%-22%) to death (0.0088%-0.3%)[2]. Liver biopsy is also limited by both inter-observer and sampling variability. Although inter-observer variability has been improved by standardized scoring systems for fibrosis and inflammation, discordance rates for the stage of fibrosis of approximately 33% have been reported[3,4]. The frequency of sampling errors is not surprising given the fact that a standard percutaneous liver biopsy specimen has been calculated to sample only 1/50 000 part of the liver[5]. Considering the expense and limitations of liver biopsy, there is great interest in the use of non-invasive markers of liver fibrosis that can replace liver biopsy both as a guide to therapy and as a prognostic indicator. Current studies have shown that non-invasive markers can predict the absence of significant disease or the presence of advanced disease at the time of liver biopsy, but it is not clear that they can predict step-wise progression of fibrosis[6]. It is also not clear if they have prognostic value regarding long-term clinical outcomes. If they can be proven to be useful longitudinally, this would be of great benefit to clinicians and their patients.

Non-invasive markers can be broadly divided into two major groups: radiological and serum-based markers. Transient elastography using ultrasound waves to measure stiffness of liver is an example of a radiological method but is limited in terms of cost, technical complexity and availability. It currently is not approved for clinical use in the United States by the Food and Drug Administration. Serum biomarkers have been proposed as an inexpensive and effective alternative to replace liver biopsy and can be subdivide into two categories: (1) indirect markers that are composed of simple routine biochemical and/or hematological tests; and (2) markers composed of substances that are part of the extracellular matrix (ECM). Although simple markers are inexpensive and available universally, the markers of ECM are expensive and available only in reference or research laboratories. Two indirect marker panels, aspartate aminotransferase (AST)/Platelet Ratio Index (APRI) and FIB-4, are composed of panels that are routinely preformed in patients with liver disease, and therefore, could be a practical and convenient way to follow patients[7,8].

Most studies of biomarkers in fibrosis have compared their diagnostic accuracy in predicting the presence of mild *vs* significant fibrosis, mild/moderate *vs* advanced fibrosis, or cirrhosis, using a liver biopsy obtained at the same time as the gold standard[6]. This approach has limitations because liver biopsy is a less than ideal reference standard and it is not clear if these biomarkers are useful in identifying patients in whom fibrosis is likely to progress. It is important to develop a non-invasive tool for predicting progression of fibrosis, because these patients are the ones who might benefit from clinical interventions like antiviral therapy and/or more frequent and intense monitoring.

In this study, we examined APRI and FIB-4 in a group of patients with paired liver biopsies to determine if longitudinal changes in the markers correlated with changes in the histological stage of fibrosis, and also examined whether the initial or follow-up APRI and FIB-4 was useful in predicting those patients who showed an increase in stage of fibrosis on liver biopsy.

MATERIALS AND METHODS

Patients studied

Following approval by the Institutional Review Board, we searched a database available in the Department of Pathology for all patients who had undergone at least two liver biopsies for staging fibrosis in mono-infected HCV-positive patients between 1999 and 2007. Patients were selected for inclusion if the liver biopsies were at least 1 year apart. Patients were excluded if they had cirrhosis, were co-infected with hepatitis B virus (HBV) or human immunodeficiency virus, had an organ transplant, daily alcohol intake of > 30 g/d, hepatocellular carcinoma or primary metabolic or autoimmune liver disease. The laboratory values used for the calculations of the APRI and FIB-4 were those that were closest to the liver biopsy date. Our hepatology service has had an ongoing prospective study of hepatic fibrosis markers since 2003, and 20 of the patients were enrolled in that study for one or both of their liver biopsies. Those patients had their blood drawn on the day of the liver biopsy. All other patients had blood work within 3 mo of the biopsy, or they were excluded from the study.

Laboratory studies

The platelets were measured using the Sysmex SE 9500 (Sysmex, Mundelein, IL, USA) and the AST and the alanine aminotransferase (ALT) were measured using either the Vitros 950 or 5,1 FS (Ortho Clinical diagnostics, Raitan, NJ, USA).

Biomarker panels

The APRI is a numerical value that is calculated using the following formula: APRI = [AST (U/L)/upper limit of normal (U/L)] × 100/platelets (10^9/L)[7]. The FIB-4 index is also a numerical value that is calculated using the following formula FIB 4 = Age × AST (U/L)/[platelets (10^9/L) × $ALT^{1/2}$ (U/L)][8].

Liver histology

All liver biopsies were read by a single pathologist (S.X.), without prior knowledge of the study status or the value of laboratory parameters being tested. Fibrosis was staged as described below. The fibrosis score is based on a five-point scale: stage 0 = no fibrosis; 1 = either mild pericellular fibrosis in the lobules, or mild portal fibrosis; 2 = periportal fibrosis, or portal fibrosis plus lobular pericellular fibrosis; 3 = septal or bridging fibrosis without evident parenchymal remodeling; and 4 = cirrhosis (with architectural remodeling and nodular formation). The rationales of including lobular pericellular fibrosis in our staging system are: (1) that this type of fibrosis is frequently observed among pa-

tients with chronic HCV infection; and (2) that zone 3 (or centrilobular) hepatic stellate cell activation is common in hepatitis C liver biopsies without other co-morbidity[9].

Statistical analysis

In the initial analysis the median AST, ALT, platelets, APRI and FIB-4 scores were compared between patients who had an increase in at least one stage on liver biopsies (progressors) and those who did not progress (non-progressors) using a non-parametric test (Mann-Whitney *U* test). Logistic regression was used to calculate the accuracy of the ALT, AST, platelets, APRI and FIB-4 in predicting progression of fibrosis. Receiver operating characteristic (ROC) curves were calculated for the biomarkers at the time of initial and repeat biopsies. Area under the ROC curve (AUROC), sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. Non-parametric tests were performed using Stats Direct statistical software (Sale, Cheshire, UK) and ROC curves were constructed using MedCalc statistical software (Marialterke, Belgium).

Calculation of sample size

Assuming that the AUROC for APRI and FIB-4 would be 0.8 if the null hypothesis is rejected and 0.5 if the null hypothesis is valid, a sample size of 29 is needed to detect the difference at an α value of 0.05 and β value of 0.8.

RESULTS

Patient characteristics

A total of 36 patients met the inclusion criteria. The baseline characteristics of the subjects are described in Table 1. The median age at the time of initial biopsies was 47 years (range: 25-68 years). Seventy-five percent (27/36) of patients were male, with 50% (18/36) Caucasians, 31% (11/36) African Americans and 19% (7/36) Hispanics. The majority (50%) of the patients were stage 1 on biopsy followed by stage 2 (25%) and stage 3 (22%). One patient had stage 0 fibrosis on biopsy, and there were no patients with cirrhosis because they were excluded from the study. The median duration between biopsies was 4 years (range: 2-9 years).

Table 1 Baseline characteristics of subjects included in the study

	n = 36
No. of males, *n* (%)	27 (75)
Age (yr, range)	47 (25-68)
Race , *n* (%)	
Caucasian	18 (50)
African American	11 (31)
Hispanic	7 (19)
Stage at time of initial biopsy, *n* (%)	
Stage 0	1 (3)
Stage 1	18 (50)
Stage 2	9 (25)
Stage 3	8 (22)
ALT (IU/mL, range)	73 (14-322)
AST (IU/mL, range)	49 (22-266)
Platelets ($\times 10^9$/L, range)	235 (185-362)
Median duration between biopsies (yr, range)	4 (2-9)

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Table 2 Differences in parameters between progressors and non-progressors at initial biopsy

	Progressors	Non-progressors	*P* value
No.	19 (14 with a 1-stage change; 5 with a 2-stage change)	17	0.999
Age (yr)	50	52	0.621
Sex	Male 13 Female 6	Male 14 Female 3	> 0.050 (NS)
APRI	0.66	0.56	0.173
FIB-4	1.307	1.347	0.268
ALT (IU/mL)	92	47	0.003
AST (IU/mL)	100	38	0.001
Platelets ($\times 10^9$/L)	235	243	0.956
Stage	2	1	0.477
Duration between biopsies (yr)	4	4	0.200

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; APRI: AST/Platelet Ratio Index; NS: Not significant.

Relationship of baseline parameters and progression

Out of a total of 36 patients, 19 (53%) had progression of fibrosis on repeat biopsies, 16 patients (44%) showed no change in stage, and one (3%) showed improvement in fibrosis on repeat biopsy. Among the patient factors examined (Table 2), the group that showed progression had significantly higher ALT and AST at baseline when compared to the group that did not show progression ($P = 0.003$ and 0.0001, respectively). There was no statistically significant difference between baseline APRI, FIB-4 index, stage at initial biopsy, age, or duration between biopsies between progressors and non-progressors.

Parameters at time of repeat biopsy

Progressors *vs* non-progressors: Differences in parameters were explored between the two groups at the time of repeat biopsies. ALT, AST, APRI and FIB-4 were significantly higher in patients who showed progression of fibrosis (Table 3). The APRI and FIB-4 at the time of the second biopsy had excellent predictive value for progression in fibrosis with an APRI $\geqslant$ 0.69 having a 79% PPV and a 78% NPV for progression of at least one stage of fibrosis. A FIB-4 $\geqslant$ 1.65 had an 81% PPV and 70% NPV for prediction of fibrosis by at least one stage. A one-stage change in fibrosis on repeat biopsies might reflect sampling variability, therefore, we examined the predictive value of the APRI and FIB-4 for a change of two stages of fibrosis. We found that the APRI and FIB-4 had excellent predictive values for a two-stage progression of fibrosis with an APRI $\geqslant$ 1.94 and FIB-4 $\geqslant$ 3.01 having a 100% PPV and NPV and 62% PPV and 100% NPV, respectively. In addition the APRI had an AUROC of 1.0 [95% confidence interval (CI): 0.85-1.00] and FIB-4 had an AUROC of 0.911 (95% CI: 0.717-0.986, $P = 0.0001$) for those who

Table 3 Differences between progressors and non-progressors on repeat biopsies

	Progressors	Non-progressors	*P* value
APRI	1.293	0.413	0.0006
FIB-4	2.816	1.36	0.0038
ALT (IU/mL)	92	47	0.0030
AST (IU/mL)	100	38	0.0001
Platelets (× 10^9/L)	184	221	0.1160

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; APRI: AST/Platelet Ratio Index.

had a two-stage change in fibrosis. The number of patients who progressed by two stages was low ($n = 5$), and caution should be exercised in interpreting this particular subset of the results.

Change in APRI and FIB-4 and progression *vs* non-progression: Changes in APRI and FIB-4 were correlated with change in stage of fibrosis. There was a significant correlation between change in stage and change in APRI ($r^2 = 0.39$, $P = 0.00001$) (Figure 1A) with the regression equation for a change in stage = 0.50 × (ΔAPRI) + 0.44. AUROCs were constructed for predictive value of change in APRI for change in stage with a ΔAPRI of 0.18 having an 80% PPV and 67% NPV in predicting progression of fibrosis. Similarly, the correlation between change in stage and ΔFIB-4 was also significant ($r^2 = 0.31$, $P = 0.00004$) (Figure 1B). The regression equation for a change in stage = 0.34 × (ΔFIB-4) + 0.32. An analysis of ROC curve revealed that a ΔFIB-4 of 0.39 had 75% PPV and NPV for predicting progression of fibrosis.

Accuracy of individual tests: AST proved to be the most useful of the tests. There was a significant correlation between change in stage of fibrosis and change in AST ($r^2 = 0.33$, $P = 0.002$). There was a less but still significant correlation of ALT between change in fibrosis and change in ALT ($r^2 = 0.21$, $P = 0.0048$). Changes in the platelets alone did not correlate well with changes in fibrosis ($r^2 = 0.07$).

DISCUSSION

Liver biopsy is the gold standard for estimating severity of fibrosis in patients with liver disease and is used as a guide for therapy of HCV infection. However, because antiviral therapy with pegylated interferon and ribavirin carries significant toxicity, and fibrosis progresses in only 20%-30% of patients with chronic HCV infection[10], therapy is ideally offered to patients whose risk of progressive liver damage is high. Current strategy involves using the stage of fibrosis on initial liver biopsy to determine the risk of progressive liver damage. However, the stage at the time of the biopsy might not predict the risk of progression, especially in a younger patient and/or someone with a shorter duration of infection.

Apart from the complications associated with the invasive nature of liver biopsy, issues like sampling variability, inter-observer variability, and expense limit its utility and diagnostic accuracy[9,11]. Factors that determine the varying rates of fibrosis progression are poorly understood, partially because of the logistical difficulties for studies in performing serial liver biopsies. Non-invasive biomarkers that reflect hepatic fibrosis have been proposed to overcome these limitations with the ideal biomarker of hepatic fibrosis being inexpensive, easily available, and accurately reflecting changes in fibrosis. The APRI and FIB-4 are two biomarker panels that incorporate readily available and routinely performed tests for all patients with liver disease. Both have been confirmed to be accurate in predicting fibrosis stage on liver biopsies[1,7,8,12-23]. However, few studies have examined the longitudinal behavior of fibrosis biomarkers. Patel *et al*[24] have evaluated a single direct hepatic fibrosis marker, hyaluronic acid (HA), in patients undergoing liver biopsies before, and 6 mo following antiviral therapy for chronic HCV. Although HA has some association with the stage of fibrosis, it does not predict histological changes over the treatment period. Our study addressed the longitudinal behavior of these markers in individuals who underwent serial biopsies, and examined the role of these biomarkers in predicting progression.

There is accumulating evidence that hepatic fibrosis markers can be used for prognosis. Following 6 mo of interferon-based therapy, an APRI > 1.5 has been shown to be associated with significant short-term mortality and risk of hepatocellular carcinoma in patients with chronic

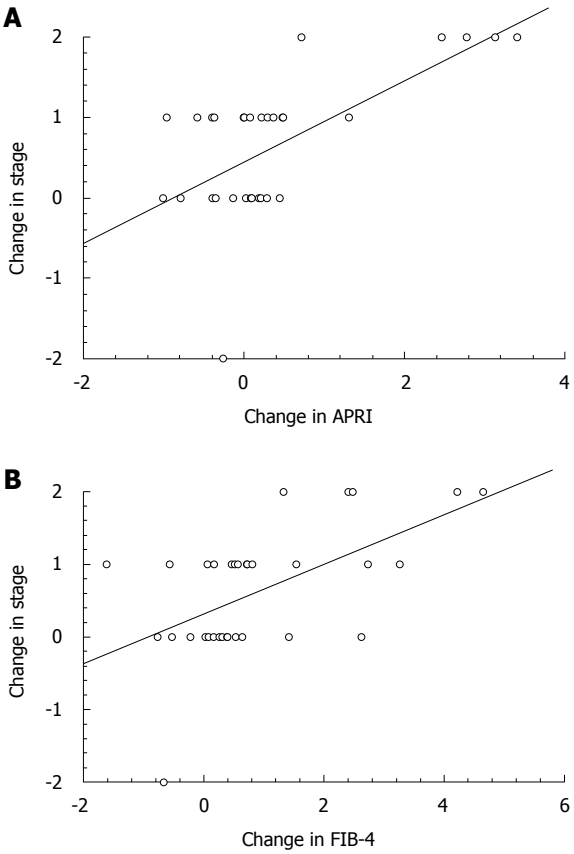

Figure 1 Linear regression plot. A: Correlation between changes in stage of fibrosis *vs* change in aspartate aminotransferase/Platelet Ratio Index (APRI); B: Correlation between stages of fibrosis *vs* FIB-4.

HCV[21]. A commercially available test, FibroTest, has been found at baseline to predict outcomes in chronic HCV[25]. In addition, two other commercially available tests, Fibrometer and Hepascore, have been shown to be as good as liver biopsy in the prediction of survival in alcoholic liver disease[26]. Recently, the European Liver Fibrosis Test has been shown to be superior to several other parameters in the prediction of outcomes of primary biliary cirrhosis[27].

One finding of our study that is potentially useful for clinicians is that serial changes in APRI and FIB-4, which are easily calculated using routine laboratory tests, correlated well with changes in fibrosis staging. There was a significant correlation between change in stage and change in APRI and FIB-4 of 0.627 ($r^2 = 0.39$, $P = 0.00001$) and 0.56 ($r^2 = 0.31$, $P = 0.00004$), respectively. A ΔAPRI of 0.18 had 80% PPV and 67% NPV in predicting progression of fibrosis, whereas a ΔFIB-4 of 0.39 had 75% PPV and NPV for predicting progression of fibrosis. These results are significant as they suggest that longitudinal changes of APRI and FIB-4 markers can be useful in predicting progression of fibrosis, which could lead to changes in clinical management.

The baseline characteristics of the study population did not differ between progressors and non-progressors, except for aminotransferases, which were significantly higher in the group that later showed progression of fibrosis, which has been shown previously[28]. This was independent of the initial stage of fibrosis and did not differ significantly between groups. It seems likely that, even with the same stage of fibrosis, higher enzyme levels are a marker for subsequent progression. The effect of timing between the biopsies is unlikely to have an impact on the conclusions because there was no significant difference between duration of biopsies between the progressor and non-progressor groups. As expected, the APRI and FIB-4 were significantly higher at the time of the second liver biopsy in the patients with progression of fibrosis, which reflected the accuracy of these tests at a single point in time. Therefore, not unexpectedly, the APRI and FIB-4 were predictive of progression at the time of the second biopsy. Although the number of patients was small, both tests were able to predict a two-stage change in fibrosis at the second biopsy.

This study had several limitations, mainly due to the largely retrospective nature of the study. Previously, we have found that the APRI was less accurate retrospectively than prospectively[16]. This might have been because the retrospective patient group was more heterogenous, as well as the fact that laboratory values were not always performed close to the time of the biopsy. In addition, concurrent illnesses that could influence aminotransferases and platelets could not be easily determined in some of the patients. Another significant limitation was the relatively small sample size. A larger and well-designed prospective trial that could confirm the role of biomarkers, including those from the ECM, in predicting fibrosis progression would be very useful.

In summary, the APRI and FIB-4 at initial liver biopsy were not useful in predicting patients whose fibrosis would be progressive. However, as previously reported[28], patients with higher aminotransferases appear to have an increased risk of progression of fibrosis. Importantly, the longitudinal use of the APRI and FIB-4 is accurate in predicting progression of fibrosis, and could be useful to clinicians that follow patients with chronic HCV. Based on our data, a ΔAPRI of 0.18 or a ΔFIB-4 of 0.39 in a patient with chronic HCV suggests progression in fibrosis of at least one stage, and could be used to trigger a reconsideration of antiviral therapy and/or liver biopsy. Larger, prospective studies are needed to validate the findings of our study.

COMMENTS

Background

The complications of chronic liver disease are largely related to the development of fibrosis. Medical treatments are aimed at prevention of progression of cirrhosis. A liver biopsy has been the usual way to estimate fibrosis. However, it is invasive and expensive, and can be inaccurate because of variability in the degree of fibrosis in parts of the liver that can be sampled. The use of certain serum biomarkers to estimate fibrosis has been found to useful, especially in chronic hepatitis C. These biomarkers can be composed of a combination of simple tests that are used to assess liver function, as well as more complex indices that measure substances in the blood that can originate in the extracellular matrix of the liver itself.

Research frontiers

Although several simple and complex biomarkers have been found to be accurate at differentiating mild from significant fibrosis, there has been little evaluation of them in a longitudinal manner. In other words, it is not clear whether changes over time in the value of the markers reflect actual changes in the degree of fibrosis. If these markers can accurately predict changes in fibrosis, then this would be useful to clinicians. Not only could this lead to fewer liver biopsies, but it could lead to changes or initiation of treatment. This study evaluated two simple biomarkers, the aspartate aminotransferase (AST)/Platelet Ratio Index (APRI) and the FIB4, longitudinally in a group of patients with chronic hepatitis who had two liver biopsies that were at least 1 year apart. It found that both biomarkers could accurately predict changes in the stage of fibrosis.

Innovations and breakthroughs

The APRI utilizes AST and platelets, which are simple tests obtained in all liver patients. It was originally described by Wai *et al* in 2003 (*Hepatology* 38: 518-526). Its usefulness has been confirmed in multiple studies including that of Snyder *et al* (*J Clin* Gastroenterol 2006; 40: 535-542). The FIB4 utilizes AST, platelets, alanine aminotransferase (ALT), and age. It was originally described by Sterling *et al* in 2006 (*Hepatology* 43: 1317-1325). A high APRI score at 6 mo following treatment has been shown to correlate with poor survival, as well as the development of hepatocellular carcinoma.

Applications

This article shows that physicians can accurately follow patients with chronic hepatic C that have failed previous antiviral therapy or that have not been treated. Treatment decisions can be based on changes in these indices. These findings should be confirmed in larger studies from other institutions. Also, further research should be done with the longitudinal use of the more complex biomarkers, as well as the longitudinal use of simple and complex biomarkers in diseases other than chronic hepatitis C.

Terminology

Liver fibrosis refers to the amount of scar tissue in the liver. This impairs blood flow and eventually can lead to cirrhosis. AST is an enzyme that is in liver cells and is released when there is active liver damage. ALT is an enzyme specific to liver cells, which is released when there is liver damage. The platelets are a component of the blood that helps with clotting. Platelets decrease with advancing liver disease and fibrosis because some of them are sequestered in the spleen, which enlarges with liver disease, and also a hormone called thrombopoeitin that stimulates platelets production is released in smaller quantities with advancing disease. Biomarkers are substances that can be objectively measured that are used as an indicator of a biological state. They are being increasingly used in medicine as non-invasive ways to screen for and evaluate various diseases including cancer.

Peer review

The authors show that evolutionary changes in simple scores (APRI and FIB-4) based on routine biochemical parameters can be of value in predicting the evolution of liver fibrosis in chronic hepatitis C. The data provided in this study have important clinical implications that pertain to the management of patients with chronic hepatitis C.

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BRIEF ARTICLE

Differential changes in intrinsic innervation and interstitial cells of Cajal in small bowel atresia in newborns

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Abstract

AIM: To investigate morphological changes of the enteric nervous system (ENS) and the interstitial cells of Cajal (ICCs) in small bowel atresia.

METHODS: Resected small bowel specimens from affected patients (n = 7) were divided into three parts (proximal, atretic, distal). Standard histology and enzyme immunohistochemistry anti-S100, anti-protein gene product (PGP) 9.5, anti-neurofilament (NF), anti-c-kit-receptor (CD117) was carried out on conventional paraffin sections of the proximal and distal part.

RESULTS: The neuronal and glial markers (PGP 9.5, NF, S-100) were expressed in hypertrophied ganglia and nerve fibres within the myenteric and submucosal plexuses. Furthermore, the submucous plexus contained typical giant ganglia. The innervation pattern of the proximal bowel resembled intestinal neuronal dysplasia. The density of myenteric ICCs was clearly reduced in the proximal bowel, whereas a moderate number of muscular ICCs were found. The anti-CD117 immunoreaction revealed additional numerous mast cells. The distal bowel demonstrated normal morphology and density of the ENS, the ICCs and the mast cells.

CONCLUSION: The proximal and distal bowel in small bowel atresia revealed clear changes in morphology and density of the ENS and ICCs.

Key words: Small bowel atresia; Enteric nervous system; Gastrointestinal motility; Interstitial cells of Cajal

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INTRODUCTION

Small bowel atresia is a congenital disorder that carries a substantial morbidity[1,2]. The etiology of bowel atresia remains unclear. One of two accepted theories of its pathogenesis is the concept of a lack of recanalization of the solid organ cord during the late stage of intestinal development[3]. Another concept is the occurrence of a late intrauterine mesenteric vascular accident[4]. The lack of revacuolization is the probable cause for most cases of duodenal atresia. Further studies have demonstrated that

jejunoileal atresias occur as a result of intestinal volvulus, intussusception, internal hernia, or strangulation in a tight gastroschisis or omphalocele defect[5-11].

Newborns with small bowel atresia are operated on soon after birth. Owing to the severity of the dilatation of the proximal bowel and the hypoplasia of the distal bowel, various postoperative gastrointestinal motility problems may occur; such problems include prolonged adynamic ileus (11%) and the need for total parenteral nutrition (30%-70%)[12]. The underlying cause of this postoperative intestinal motility disorder is still unclear.

Basically, normal gastrointestinal motility depends on the coordinated function of the enteric nervous system (ENS), the intestinal smooth muscle and the interstitial cells of Cajal (ICCs). Previous studies revealed histological changes within the wall of the atretic and adjacent bowel in small bowel atresia. Hypertrophy of the bowel muscle proximal to the atresia was found in clinical and experimental studies on small bowel atresia. Various changes have been reported within the ENS in small bowel atresia[13]. Nevertheless, the relationship between the macroscopic and histological changes of the affected bowel and the postoperative motility disorder are still under investigation. Furthermore, the role of the ICCs in small bowel atresia needs to be elucidated further.

ICCs play a major role in gastrointestinal motility. ICCs express the tyrosine kinase receptor c-kit. Therefore specifically designed c-kit antibodies have been developed which stain ICCs but also other cell groups such as stem cells and mast cells. However, c-kit positive ICCs can be identified by clear morphological features.

The aim of this study was to investigate the morphology of the ENS and the ICCs in resection specimens of small bowel atresia.

MATERIALS AND METHODS

Patients and tissues

Resected small bowel specimens (ileum) from affected newborn patients (n = 7) were included in the investigation after parental consent. The resected ileal specimens were divided into three parts (proximal, atretic, distal).

Tissue processing

The specimens were fixed in 4% paraformaldehyde and processed into paraffin blocks. Paraffin-embedded tissues were sectioned at 2-4 μm (Leica SM 2000 R) followed by drying at 37℃ in an incubator overnight. Before immunohistochemical staining, the paraffin sections were dewaxed for 10 min in xylene, followed by 10 min in acetone and 10 min in acetone/Tris-buffered saline (TBS; 1:1). After this treatment, the slides were washed in TBS.

Antigen demasking

If heat antigen retrieval was required, dewaxed paraffin sections were placed in microwave-proof tubes containing target retrieval solution (Dako). The slides were treated in the tubes for 5 min at 600 W in a microwave (SS 566H; Bosch, Munich, Germany). The evaporated volume was replaced by distilled water, and the procedure was repeated twice. After microwave treatment, the slides were left to cool down, then were washed in TBS.

Table 1 Primary antibodies

Antibody	Clone	Company	Dilution
S-100	Polyclonal (Z0311)	Dako, Glostrup, Denmark	1:500
PGP-9.5	Polyclonal (Z5116)	Dako, Glostrup, Denmark	1:25
Neurofilament	2F11 (M0762)	Dako, Glostrup, Denmark	1:50
c-kit	Polyclonal (A4502)	Dako, Glostrup, Denmark	1:50

PGP: Protein gene product.

Histochemistry and immunohistochemistry

Standard histology [hematoxylin/eosin (HE)] was performed on the sections. For immunohistochemistry, an alkaline phosphatase-anti-alkaline phosphatase (APAAP) staining kit (Dako Real™ Detection System, APAAP, Mouse) using anti-S100, anti- protein gene product (PGP) 9.5, anti-neurofilament (NF) and anti-c-kit-receptor (CD117) antibodies was employed. A non-sense mAb (clone: MR 12/53) served as negative control by omitting the primary antibody. The final concentrations are given in Table 1.

Evaluation

The evaluation of the immunohistochemical staining results was focused on the proximal and distal parts of the resected ileum.

The sections were evaluated by two independent investigators using light microscopy (magnification, × 40). HE staining was used to determine the overall histology of the investigated specimen. The distribution and density of immunoreactive ganglion cells, glial cells, nerve fibers and c-kit positive cells (ICCs, mast cells) were studied in each part of the resected bowel (proximal, atretic, distal). Since quantitative analysis of immunohistochemical staining is not possible, semi-quantitative scoring was performed as follows; - no expression (no staining), + low expression (few neuronal fibers and cells/few ICCs), ++ moderate expression (numerous neuronal fibers and cells/numerous ICCs), +++ high expression (dense networks of neuronal fibers and cells/dense network of ICCs).

RESULTS

Patients

The study included resected ileal segments from seven newborn patients (gestational age 38-40 wk). Six patients presented with type Ⅲa ileal atresia and one patient presented with multiple ileal atresia.

Neuronal markers (PGP 9.5, NF)

The neuronal markers PGP 9.5 and NF were strongly expressed within the ganglion cells and nerve fibers of the myenteric plexus (and submucosal plexus in the proxi-

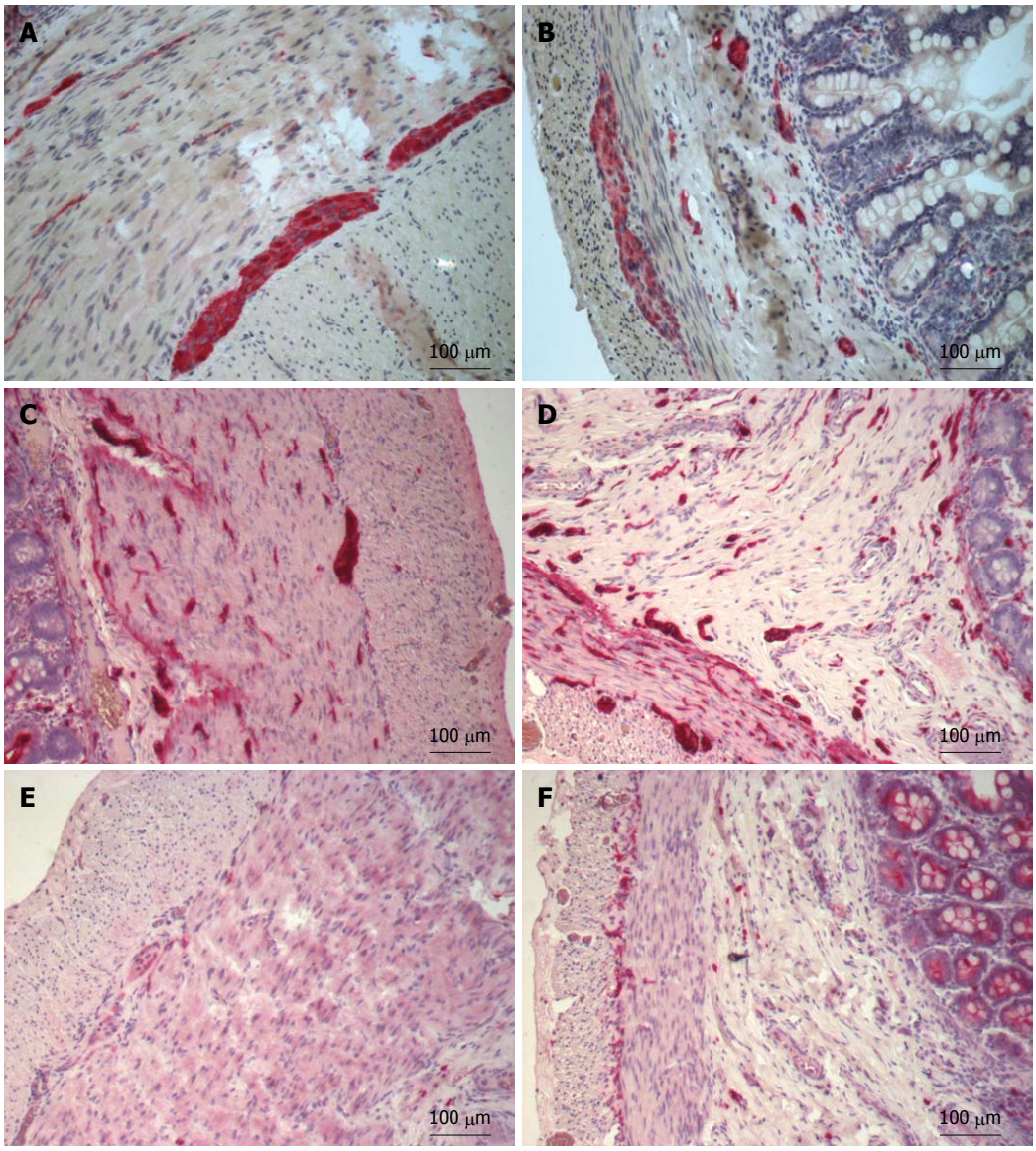

Figure 1 Immunohistochemistry of a full-thickness section of the ileum. A: Protein gene product (PGP) 9.5 in the ileum proximal to the atresia shows a dense innervation within the submucosal and myenteric plexus; B: PGP 9.5 in the ileum distal to the atresia shows a normal innervation pattern of the submucosal and myenteric plexuses; C: S-100 in the ileum proximal to the atresia shows numerous fibers and large ganglia (> 20 μm) within the submucosal plexus and normal innervation within the myenteric plexus; D: S-100 in the ileum distal to the atresia shows a normal innervation pattern within the submucosal and myenteric plexuses; E: c-kit in the ileum proximal to the atresia shows that myenteric and muscular interstitial cells of Cajal (ICCs) are almost absent; F: c-kit in the ileum distal to the atresia shows normal distribution of myenteric and muscular ICCs.

Table 2 Distribution and density of immunostaining (proximal *vs* distal)

	Proximal				Distal			
	SP	CM	MP	LM	SP	CM	MP	LM
PGP 9.5	++	+	++	+	++	+	++	+
S-100	+++	+	+++	+	++	+	++	+
NF	++	+	++	+	++	+	++	+
c-Kit (ICCs)	-	-/+	+/-	-	-	+	++	+

PGP: Protein gene product; ICCs: Interstitial cells of Cajal; SP: Submucosal plexus; CM: Circular muscle; MP: Myenteric plexus; LM: Longitudinal muscle; NF: Neurofilament; -: No expression (no staining); +: Low expression (few neuronal fibers and cells/few ICCs); ++: Moderate expression (numerous neuronal fibers and cells/numerous ICCs); +++: High expression (dense networks of neuronal fibers and cells/dense ICCs networks).

mal ileum (Figure 1A). The submucosal plexus contained some giant ganglia (n > 10 ganglion cells) and numerous thick nerve fibers. The overall numbers of ganglia were not increased within the myenteric and submucosal plexuses (Table 2). The immunohistochemical staining revealed features of intestinal neuronal dysplasia (giant ganglia, hypertrophied nerve fibers).

The distal bowel had a normally expressed ENS within the submucosal and myenteric plexuses (Figure 1B).

Glial marker (S-100)

Numerous S-100 immunoreactive glial cells were found in the two plexuses of the proximal ileum (Figure 1C). The expression of glial cells was increased within the proximal dilated bowel compared to the atretic and distal bowel

(Table 2). Additional giant ganglia (> 10 ganglion cells/ganglion) were stained by S-100 within the submucosal plexus of the proximal bowel. Regular distribution and morphology of S-100 immunoreactive cells and fibers was found within the myenteric and submucosal plexuses of the distal ileum (Figure 1D).

c-kit (CD-117) staining (ICCs, mast cells)

The density of ICCs within the myenteric plexus was clearly reduced in the proximal bowel (Figure 1E), whereas some ICCs were found in the circular and longitudinal muscle layer (Table 2). The anti-CD117 immunoreaction revealed numerous mast cells within the proximal bowel. The distal bowel had a normal distribution of ICCs within the myenteric plexus and muscular layers and a normal number of mast cells (Figure 1F).

DISCUSSION

This study revealed distinct changes in the morphology of the ENS and ICCs in parts of the resected bowel proximal to small bowel atresia. In general, our findings are not unique since previous studies described changes within the ENS in small bowel atresia. Ozguner *et al*[14] reported that the proximal segment of the atretic intestine showed structural deficits. Abnormal ganglia cells and defects in the intestinal musculature were prominent, but the intestinal mucosa remained intact. They found abnormalities in both the antimesenteric side and mesenteric side. Their interpretation supported a vascular accident as a causative factor.

Di Nardo *et al*[15] presented an interesting case report of small bowel atresia that was initially diagnosed as hyper-ganglionosis and later changed to an enteric degenerative neuropathy. Watanabe *et al*[16] contributed an elegant study investigating the myenteric plexus in small bowel atresia using whole-mount preparations. This study showed mild hypoplasia of the ENS plexuses in the proximal segments[16].

In our study the expression of nerve fibers and glial cells appeared to be slightly increased, and some submucosal giant ganglia were found, which resembled intestinal neuronal dysplasia within the proximal dilated bowel. The expression of c-kit positive ICCs was clearly decreased in the dilated proximal bowel. The reduction of ICCs within the proximal dilated bowel has also been described before. In contrast to our study, some previous investigators have shown a reduction of the innervation density within the proximal bowel segments[13]. The innervation pattern and the expression of ICCs within the distal small bowel were normal in our study, similar to comparable investigations[13].

The crucial problem of investigating the developing ENS or pathological changes within the ENS in order to define hyper-ganglionosis or hypoganglionosis is the uncertainty in the normal numbers and distribution of enteric ganglia. The normal neuron density of the human myenteric plexus was investigated in several studies and resulted in a huge variation of more than 200-fold[17-20]. Schuffler *et al*[17] found 27.8 nerve cells per 100 mm of smooth muscle, Ikeda *et al*[19] described more than 30 nerve cells per 10 mm of smooth muscle as normal, whereas Smith[18] estimated 7 nerve cells per mm of smooth muscle. Finally Meier-Ruge *et al*[20] found 756 ganglion cells per 10 mm of smooth muscle. All these investigations were performed on conventional paraffin sections[17-20], which usually display only a two-dimensional image of the investigated structures. Since these profound differences have already been found within normal bowel specimens, conflicting results on numerical changes of ganglia and nerve fibers within the changed ENS in bowel atresia must be expected. Another major aspect is the widely accepted concept of ongoing postnatal plasticity of the ENS, which has to be taken into consideration.

A previous animal study which created a partial obstruction in dog ileum revealed that the ganglion cells increased in size and the smooth muscle of the dilated bowel became thicker[21]. The number of ganglion cells related to the muscle mass was decreased above the constriction. Although some ganglion cells increased in size, there was no evidence of any increase in number. Experimentally, a similar increase in ganglion size was observed proximal to a stenosis in another study[22]. In a chicken embryonic model of bowel obstruction, a marked reduction of the submucous plexus ganglia with intact muscular plexus ganglia was noted in the dilatated gut[23].

Our study also showed an increase in size of ganglia (hypertrophy). These hypertrophic myenteric and submucosal ganglia in the proximal resected bowel could be considered the result of adaptive phenomena to the long-lasting prenatal bowel obstruction. Several experimental models have been instrumental in supporting the concept that adaptive changes to the ENS occur proximal to the obstructed gut[24-31]. These studies revealed an increased density and size in both myenteric[26,28,29,31] and submucosal neurons[29-31], along with neurochemical[28,30] and cytoskeletal abnormalities of the myenteric neurons[31].

Ekblad *et al*[28] showed that, in the hypertrophic ileum, several neuronal populations showed changes in the expression of neuro-messengers. Myenteric neurons expressing vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide, and galanin were notably increased in number. In submucosal ganglia, the number of VIP-immunoreactive neurons decreased, while those expressing VIP mRNA increased. NADPH diaphorase-positive submucosal neurons increased dramatically, while the number of neuronal-type nitric oxide synthase expressing neurons was unchanged. The number of ICCs decreased markedly in the hypertrophic ileum.

Another observation is the delayed maturation of ENS within the distal bowel during fetal development in cases with small bowel atresia[32]. Distortion of the polygonal architecture of the myenteric plexus has been shown postnatally in atretic parts of ileal type IIIa atresia[33]. It could only be speculated that after the restoration of bowel continuity does maturation of the ENS occur.

A previous animal study using a partial small obstruc-

tion model in mice revealed that 2 wk following the onset of a partial obstruction, the bowel increased in diameter, and hypertrophy of the tunica muscularis was observed oral to the obstruction site[27]. Networks of ICCs were disrupted oral to the obstruction, and this disruption was accompanied by the loss of electrical slow waves and responses to enteric nerve stimulation. These defects were not observed aboral to the obstruction. Furthermore, it was shown that removal of the obstruction led to the redevelopment of ICC networks and the recovery of slow wave activity within 30 d. Neural responses were partially restored in 30 d[27]. Similar repair mechanisms may occur after surgical correction of small bowel atresia. It seems obvious that decreases in ICCs in small bowel atresia and their restoration after removal of the obstruction contributes to the regulation of gastrointestinal motility.

Masumoto *et al*[34] showed muscular alterations in both segments of IA cases, which further contributes to the postoperative motility disorder. Another case report recently revealed long lasting chronological changes within the ENS, muscle components and ICCs in small bowel atresia[35].

The ENS and ICCs are altered in the proximal and dilated bowel in small bowel atresia. The innervation pattern of the proximal bowel resembles intestinal neuronal dysplasia. These changes might be the result of long-lasting bowel obstruction and stasis of bowel contents. The presented histological features do not contribute to the search for the pathogenesis of small bowel atresia. Nevertheless, the confirmed innervation abnormalities and defective expression of CD117-positive cells of the proximal bowel may have an influence on the postoperative gastrointestinal motility of the affected patients.

COMMENTS

Background

Small bowel atresia is a congenital anomaly of unknown cause. Despite early corrective surgery, patients carry a substantial morbidity because of postoperative gastrointestinal motility problems. Normal gastrointestinal motility is generated by the complex interaction of the enteric nervous system (ENS), the intestinal smooth muscle and the interstitial cells of Cajal (ICCs). Alterations in the ENS and ICCs may contribute to the motility problems in patients with small bowel atresia after surgery.

Research frontiers

Previous studies revealed histological changes within the wall of the atretic and adjacent bowel in small bowel atresia. Nevertheless the relationship between the macroscopic and histological changes of the affected bowel and the postoperative motility disorder are still under investigation. Furthermore, the role of the ICCs in small bowel atresia needs to be elucidated further.

Innovations and breakthroughs

This study showed that the ENS and ICCs are altered in the proximal and dilated bowel in small bowel atresia. The innervation pattern of the proximal bowel resembles intestinal neuronal dysplasia.

Applications

The changes within the ENS and the ICCs may be the result of a long-lasting bowel obstruction and stasis of bowel contents. The presented histological features do not contribute to the search for the pathogenesis of small bowel atresia. However, the confirmed innervation abnormalities and defective expression of CD117-positive cells of the proximal bowel may have an influence on postoperative gastrointestinal motility in the affected patients.

Terminology

Bowel atresia is a congenital defect in the continuity of the bowel. The incidence of small bowel atresia is higher than that of large bowel atresia and varies between 1:300 to 1:3000.

Peer review

The authors studied the ileum of seven patients with ileal atresia. They reported that the proximal dilated intestine had an increased expression of nerve fibers and glial cells, with a decrease in ICCs, and an increase in ganglia size, whereas the distal intestine displayed normal features. They conclude that these alterations may influence on postoperative intestinal motility. The authors performed a well designed study in a very uncommon congenital disease, which is very interesting for the readers.

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BRIEF ARTICLE

Esophagogastric anastomosis with invagination into stomach: New technique to reduce fistula formation

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Abstract

AIM: To present a new technique of cervical esophagogastric anastomosis to reduce the frequency of fistula formation.

METHODS: A group of 31 patients with thoracic and abdominal esophageal cancer underwent cervical esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach tube. In the region elected for anastomosis, a transverse myotomy of the esophagus was carried out around the entire circumference of the esophagus. Afterwards, a 4-cm long segment of esophagus was invaginated into the stomach and anastomosed to the anterior and the posterior walls.

RESULTS: Postoperative minor complications occurred in 22 (70.9%) patients. Four (12.9%) patients had serious complications that led to death. The discharge of saliva was at a lower region, while attempting to leave the anastomosis site out of the alimentary transit. Three (9.7%) patients had fistula at the esophagogastric anastomosis, with minimal leakage of air or saliva and with mild clinical repercussions. No patients had esophagogastric fistula with intense saliva leakage from either the cervical incision or the thoracic drain. Fibrotic stenosis of anastomoses occurred in seven (22.6%) patients. All these patients obtained relief from their dysphagia with endoscopic dilatation of the anastomosis.

CONCLUSION: Cervical esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach tube presented a low rate of esophagogastric fistula with mild clinical repercussions.

Key words: Esophageal cancer; Esophagectomy; Constriction; Pathologic; Fistula; Gastroplasty

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INTRODUCTION

Definitive curative treatment for cancer of the esophagus remains a challenge for surgeons[1-8]. The approach involves major surgery that has high morbidity and mortality rates, mainly due to pulmonary complications,

cervical fistulas, stenosis of anastomosis, necrosis of the tubularized stomach, and mediastinitis[5,9-12].

Among these possible complications, fistula of the esophagogastric anastomosis represents one of the principal problems of esophagectomy. In several studies, the incidence has ranged from 0% to 50%, with most authors reporting a high incidence of this complication[4,6,13-18].

Although these fistulas usually have a favorable course, about 2% of cases can have a catastrophic outcome[5,16]. In cases in which the fistula does not lead directly to death, it can compromise quality of life, interfere with resumption of feeding, require laborious local care, and prolong hospital stay. Additionally, 30%-50% of those patients who present with fistula go on to develop stenosis[15,19-22].

Given this scenario and personal experience of a high incidence of cervical esophagogastric fistula in treatment of carcinoma of the esophagus[23], we decided to perform cervical esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach tube, and to analyze the incidence of fistula and stenosis formation following this procedure.

MATERIALS AND METHODS

This study conformed to the regulations of The Human Ethics Research Committee at our Institution and with the Helsinki Declaration, revised in 1983.

Our study group consisted of 31 patients with thoracic or abdominal carcinoma of the esophagus, who underwent open access with subtotal esophagectomy and esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach tube. The study group included 27 (87.1%) men and four (12.9%) women, with a mean age of 60.2 ± 8.5 years (range: 44-74 years). Lesions were located in the medial third of the esophagus in 15 cases (48.3%) and the inferior third in 16 (51.6%).

The inclusion criteria for operation were: esophagogram with no abnormal axis deviation, lesions up to 5.0 cm long, absence of signs of invasion of the respiratory tree on bronchoscopy, and absence of signs of irresectability of the esophageal lesion or neoplastic dissemination on thoracic and abdominal helicoidal tomography. Cases in which an anesthetic or surgical procedure was contraindicated due to compromised clinical state and/or concurrent serious systemic disease were excluded from the study. The diagnosis was confirmed by upper esophageal endoscopy and biopsy: 25 (80.6%) patients had squamous cell carcinoma and the remaining six (19.3%) had adenocarcinoma.

All patients underwent preoperative clinical evaluation. Thirteen (41.9%) had serious clinical malnutrition as shown by weight loss of greater than 20% of normal weight. Tumor staging was performed using physical examination, thoracic radiography, barium esophography, thoracic and abdominal tomography, and bronchoscopy in patients whose lesions were situated in the medial third of the esophagus. Clinicopathological staging using the TNM classification by the UICC[24] was: stage I in two (6.4%) patients; stage ⅡA in five (16.1%); stage ⅡB in four (12.9%); stage Ⅲ in 16 (51.6%); and stage ⅣA in four (12.9%).

Surgical technique

In the absence of contraindications, a transhiatal esophagectomy followed by a cervical esophagogastroplasty was performed. All surgeries were carried out in parallel during the same operating period by two teams, with one team operating in the abdominal region and the other in the cervical region. Lymph node resection was done in both the abdominal and inferior mediastinal fields. In all cases, the tubularized stomach was placed into the cervical region by the posterior mediastinum.

The esophagus was dissected and separated from its neighboring structures in the cervical, thoracic and abdominal areas. In distally located tumors, the esophagus was sectioned in the cervical region, with care taken to preserve enough of the proximal end to allow 4.0 cm of esophagus to be inserted into the stomach, with a safe margin ⩾ 5.0 cm. The esophagus was then pulled to the abdominal region, and the stomach sectioned with a linear stapler that released the surgical specimen.

To guarantee a sufficient margin for lesions that involved the middle third, the stomach was initially sectioned and tubularized, and the piece pulled up to the cervical region, where it was very carefully examined, and the surgical section site was chosen with a safe margin. If the margin was judged to be inadequate, end-to-end anastomosis was performed instead of invagination, and that patient was excluded from the study.

In the region that was selected for anastomosis, a transverse myotomy was carried out around the entire circumference of the esophagus (Figure 1A). The proximal border of the myotomy was anastomosed, with the tip of the tubularized stomach placed in the cervical region. The anastomosis of the posterior wall was performed first using interrupted sutures of 4-0 polydioxanone (Figure 1B). Subsequently, the 4-cm segment of esophagus was introduced or invaginated into the stomach and sutured to the anterior wall as per the posterior wall (Figure 1C). In all patients, extra-mucosal pyloroplasty was carried out, a nasogastric tube was also inserted, and the cervical region was drained by a laminar drain.

Oral feeding was typically started on the postoperative day 10, in the absence of signs of esophagogastric fistula. If a fistula was present, the affected site was treated, to maintain feeding by nasogastric tube. In this case, oral diet was begun following closure of the fistula.

RESULTS

No patients died intraoperatively. Postoperative minor complications occurred in 22 (70.9%) patients. Four (12.9%) patients had serious complications that led to death: two (6.4%) as a result of bronchopneumonia, one due to multiple organ failure after acute cholecystitis, and the other from sepsis following ischemic necrosis of the stomach; all of them with no relationship to the esophagogastric anastomosis.

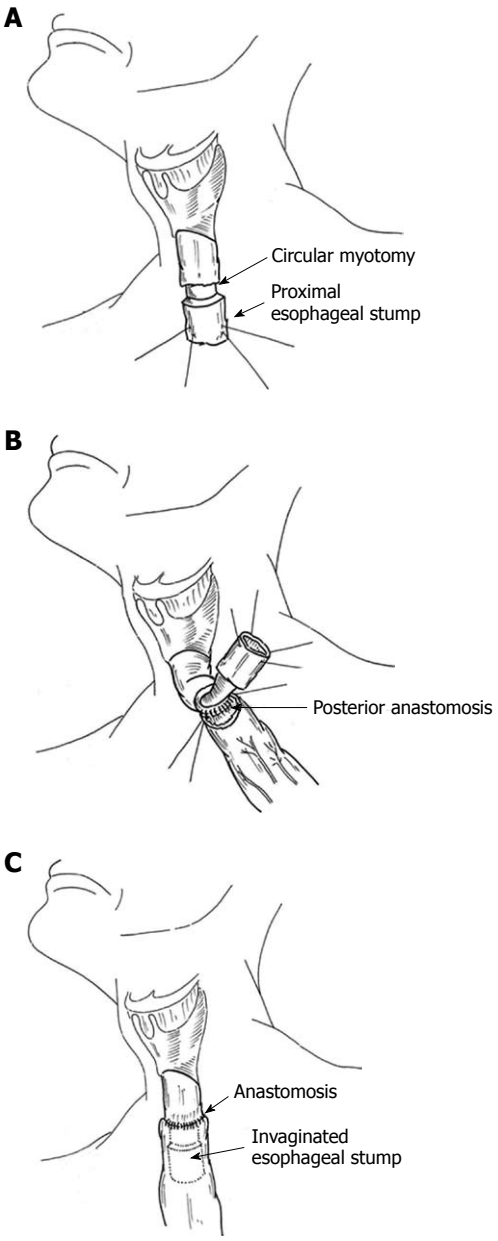

Figure 1 Esophagogastric anastomosis with stomach invagination. A: Diagram showing the circular myotomy (long arrow) in the section of the proximal esophageal stump (short arrow), which created a 4.0 cm segment of extension to be invaginated into the stomach (the illustration of the trachea was omitted); B: Diagram showing the anastomosis of the posterior wall of the esophagus performed first using interrupted sutures (the illustration of the trachea was omitted); C: Diagram showing the sectioned esophagus protruding into the stomach (the illustration of the trachea was omitted).

Three (9.7%) patients had fistula at the esophagogastric anastomosis with minimal leakage of air or saliva; all of them with mild clinical repercussions. Two of these had a fistula on postoperative days 7 and 10, with the leak of a small quantity of air or saliva from the cervical incision and consequent formation of a bubble during swallowing. In these two patients, spontaneous closure occurred after 10 and 5 d, respectively. The third case had a seropurulent pleural effusion on postoperative day 13, which was later drained. There was a negligible quantity of secretion from the pleural drain, which indicated a mild

Table 1 Postoperative complications in 31 patients with thoracic and abdominal esophagus cancer, who underwent cervical esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach tube

Complication	*n* (%)
Dysphonia	15 (48.4)
Anastomosis stricture	7 (22.6)
Bronchopneumonia	6 (19.3)
Anastomosis fistula	3 (9.7)
Atelectasis	2 (6.4)
Renal failure	2 (6.4)
Gastric ischemic necrosis	1 (3.2)
Acute cholecystitis	1 (3.2)
Wound infection	1 (3.2)

blocked esophago-pleurocutaneous fistula. This patient had no adverse effects from this and was discharged from hospital on postoperative day 23. No patients had esophagogastric fistula with intense saliva leakage from either the cervical incision or the thoracic drain.

Postoperative stricture of the anastomosis occurred in seven (22.6%) patients; in six of these, this appeared within 16-60 d, and the last appeared at 12 mo after surgery. All of these patients obtained relief from their dysphagia with endoscopic dilatation of the anastomosis, with the number of sessions required ranging from one to seven (mean = 3). One (3.2%) patient with a lesion located in the inferior third of the esophagus had recurrence of the cancer in the area of the anastomosis and required a nasogastric tube. The other complications were successfully treated: dysphonia in 15 (48.4%), bronchopneumonia in four (12.9%), atelectasis in two (6.4%), renal failure in two (6.4%), and wound infection in one (3.2%). Dysphonia was temporary and resolved after a few weeks in all cases. Patients with pre-renal renal failure responded well to expansion with fluids. Atelectasis was reversed with respiratory physiotherapy. The mean length of hospital stay was 15.2 d, with a range of 13-35 d (Table 1).

DISCUSSION

Esophagogastric anastomosis with invagination is a modification of a technique that is performed to reduce fistula formation at the anastomosis site[22]. Szücs *et al*[22] have reported 108 patients that underwent esophagectomy with esophagogastric anastomosis and telescoping of a 10-15-mm length of the esophageal end into the stomach. Twelve (11.1%) of these patients developed fistula at the anastomotic site. We chose to invaginate a 4.0-cm segment made up of all the layers of the esophagus wall, a much longer segment than that suggested by Szücs *et al*[22] and we added a transverse myotomy around the circumference of the esophagus. Our intention was not only to cover the entire site of the anastomosis, but also to encourage the discharge of saliva at a lower region, while attempting to leave the anastomosis site out of alimentary transit. To this end, it was necessary to invaginate a longer segment that consisted of all the layers of the wall of the esopha-

gus, such that the inserted portion remained in the shape of a tube in the interior of the stomach.

To execute the anastomosis, we elected a region at the proximal esophagus where the suture would be placed, and preserved 4.0 cm of esophagus to be invaginated into the stomach. At this point, a transverse myotomy was done around the circumference of the esophagus. We sutured the proximal border of the myotomy together with the seromuscular layer of the stomach. The purpose of the myotomy was to create a border with viability in the muscular layer of the esophagus, to be sutured with the seromuscular layer of the stomach, and also to elongate the esophageal tube to be inserted into the stomach.

The point of esophageal section must be chosen to allow for a safe margin, because carcinoma of the esophagus can disseminate within the wall to sites distal from the principal lesion[8,25-28]. To perform an esophagogastric anastomosis with invagination, it is necessary to save 4.0 cm more of proximal esophagus than for anastomosis without invagination. If it is not possible to achieve an adequate margin, the invagination procedure should be abandoned.

In our view, the fact that this technique conserves 4.0 cm more of the esophagus does not detract from the radical nature of the operation. Upon constructing a cervical esophagogastric anastomosis with invagination, the amount of remaining esophagus is no greater than that usually left when anastomosis is done in the thoracic apex. Walther *et al*[6], in a prospective randomized study, have compared cervical with intrathoracic esophagogastric anastomosis. They have concluded that the withdrawal of an extra 5.0 cm of esophagus to perform anastomosis in the neck does not affect the 5-year survival rate. Consequently, we believe that following all the recommendations and leaving a secure margin, esophagogastric anastomosis with invagination does not breach any radical oncological principles.

The diagnosis of fistula of the esophagogastric anastomosis was made based exclusively on clinical criteria, given that a radiological study with water-soluble contrast medium has low sensitivity and a high incidence of false-negative results[29]. None of our cases operated upon by esophagectomy with esophagogastric anastomosis and invagination developed fistula with heavy egress of saliva from the cervical incision. Compared with results from the literature[4,6,13-18,30] which show an incidence of fistulas of 0%-50%, cervical esophagogastric anastomosis with invagination had a low incidence of fistula formation, with only one case (3.4%) having clinical repercussions.

It is possible that esophagogastric anastomosis with invagination did not influence the factors responsible for the formation of the fistula. Moreover, it is likely that points of dehiscence could occur along the suture line similarly when we perform the end-to-end technique. However, as the saliva flows to an area below the anastomosis, these points of dehiscence probably can undergo rapid regeneration. On the other hand, in cases without invagination, the saliva discharges directly into the area of the suture with dehiscence, which provokes local inflammation and infection, thereby delaying the healing process of the suture line and enlarging this area.

In view of this mechanism, we believe that the three cases observed of fistula formation in esophagogastric anastomosis presented with mild clinical repercussions, even when the fistula was directed toward the pleural space. We also believe that, despite fistula with minimal clinical repercussions, the technique of esophagogastric anastomosis with invagination can still prove advantageous over the method without invagination.

In the present study, seven (24.1%) cases developed postoperative strictures of anastomosis; this rate lies within the 5%-45% limit described by other authors[15,19,20]. We believe that this result could have been due to the fact that anastomosis with invagination did not influence the factors that might predispose the formation of fistulas, such as ischemia in the proximal portion of the gastroplasty. In this situation, the points of dehiscence would have occurred along the suture line at a similar rate to anastomosis without invagination. However, the presence of a fistula was not always identified using clinical criteria, possibly due to the fact that saliva discharges below the point of the dehiscence. These events could possibly trigger a fibrotic reaction and scarring, with subsequent stenosis formation in the anastomosis.

We conclude that performing cervical esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach in subtotal esophagogastrectomy with gastroplasty in patients with carcinoma of the thoracic and abdominal regions of the esophagus represents a potential real advantage of this technique over other conventional techniques without invagination.

However, prospective, randomized, and controlled studies that involve esophagogastric anastomosis with invagination of the proximal esophageal stump into the tip of the stomach placed to the cervical region are needed to confirm the initials results obtained in this study.

COMMENTS

Background

Definitive curative treatment for cancer of the esophagus remains a challenge for surgeons. The approach involves major surgery that has a high morbidity and mortality rate, mainly due to pulmonary complications, cervical fistulas, stenosis of anastomosis, necrosis of the tubularized stomach, and mediastinitis. Among these possible complications, fistula of the esophagogastric anastomosis represents one of the principal problems of esophagectomy. Incidence in several studies has ranged from 0% to 50%, with most authors reporting a high incidence of this complication.

Research frontiers

In view of the high incidence of esophagogastric fistulas associated with significant levels of mortality and morbidity, several surgical techniques have been tried to reduce the frequency of fistula formation. These approaches include protection of the anastomosis with fibrin glue, anastomosis in two stages, gastric fundus rotation, microsurgical revascularization of the transposed viscera, mechanical anastomosis, laparoscopic construction of the gastric tube 5 d before esophagectomy, preservation of the vascular arcade of the splenic hilum, administration of prostaglandin E1, and anastomosis with invagination.

Innovations and breakthroughs

Esophagogastric anastomosis with invagination is a modification of a technique that is performed to reduce fistula formation at the anastomosis site. We chose to invaginate a 4.0-cm segment made up of all the layers of the esophagus wall, a much longer segment than that suggested by other authors, and we added transverse myotomy around the circumference of the esophagus. Our intention was not only to cover the entire site of the anastomosis, but also to

encourage the discharge of saliva at a lower region, while attempting to leave the anastomosis site without contact with saliva. To this end, it was necessary to invaginate a longer segment that consisted of all the layers of the wall of the esophagus, such that the inserted portion remained in the shape of a tube in the interior of the stomach.

Applications

Cervical esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach in subtotal esophagogastrectomy with gastroplasty in patients with carcinoma of the thoracic and abdominal regions of the esophagus is associated with a low incidence of esophagogastric fistula, while having similar stenosis rates to anastomosis without invagination.

Terminology

Cervical esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach tube is a new technique of cervical esophagogastric anastomosis to reduce the frequency of fistula formation.

Peer review

This is an interesting report on a novel technique for cervical esophagogastric anastomosis after esophagectomy and gastric replacement.

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BRIEF ARTICLE

SLC11A1 polymorphisms in inflammatory bowel disease and *Mycobacterium avium* subspecies *paratuberculosis* status

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Abstract

AIM: To test for association of *SLC11A1* with inflammatory bowel disease (IBD) and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) status in a Caucasian cohort.

METHODS: Five hundred and seven Crohn's disease (CD) patients, 474 ulcerative colitis (UC) patients, and 569 healthy controls were genotyped for *SLC11A1 1730G>A* and *SLC11A1 469+14G>C* using pre-designed TaqMan® SNP assays. χ^2 tests were applied to test for association of single nucleotide polymorphisms (SNPs) with disease, and the presence of MAP DNA.

RESULTS: *SLC11A1 1730G>A* and *SLC11A 1469+14G>C* were not associated with CD, UC, or IBD. The *SLC11A1 1730A* minor allele was over-represented in patients who did not require immunomodulator therapy (P = 0.002, OR: 0.29, 95% CI: 0.13-0.66). The frequency of the *SLC11A1 469+14C* allele was higher in the subset of study participants who tested positive for MAP DNA (P = 0.02, OR: 1.56, 95% CI: 1.06-2.29). No association of *SLC11A1 1730G>A* with MAP was observed.

CONCLUSION: Although *SLC11A1* was not associated with IBD, association with MAP suggests that *SLC11A1* is important in determining susceptibility to bacteria implicated in the etiology of CD.

Key words: *NRAMP1*; Crohn's disease; Ulcerative colitis; IS900 polymerase chain reaction

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INTRODUCTION

The solute carrier family 11 (*SLC11A1*) gene (also known

as *natural resistance associated macrophage protein 1*, *NRAMP1*)[1] has been associated with susceptibility to intracellular pathogens since its initial identification in mice[2]. *SLC11A1* encodes a divalent cation transporter that is located in endosome and phagosome membranes[3] of macrophages and monocytes within the liver, spleen and lungs[2,4]. This transporter plays a key role in mounting an effective immune response against intracellular pathogens[1,5] through its involvement in the acidification of the phagosomes[6], as well as the regulation of nitric oxide, interleukin-10[7] and vacuolar iron concentrations[8].

Given the pivotal roles that *SLC11A1* plays in innate immunity, it is not surprising that the relationship between polymorphisms in *SLC11A1* and a number of autoimmune and mycobacterial diseases has been explored. Associations have been found with leprosy[9], tuberculosis[10], rheumatoid arthritis[11], visceral leishmaniasis[12], multiple sclerosis[13], type 1 diabetes mellitus[14], and inflammatory bowel disease (IBD)[15-18]. Most of these disease associations have been with a promoter dinucleotide microsatellite (GT_n) that is known to affect *SLC11A1* expression levels[19]. However, *SLC11A1* also contains a number of single nucleotide polymorphisms (SNPs), including *SLC11A1 1730G>A* (*rs17235409*; D543N) and *SLC11A1 469+14G>C* (*rs3731865*; INT4G>C). The non-synonymous SNP 1730G>A is thought to alter the protein function[18], whereas the intronic SNP 469+14G>C has no known functional effect, but has been suggested to be in linkage disequilibrium with functional promoter polymorphisms[12].

SLC11A1 1730G>A and *SLC11A1 469+14G>C* have been tested for association with Crohn's disease (CD) in two European cohorts. Although the smaller of the two studies found no association with CD, Gazouli *et al*[18] have reported a significant association of both SNPs with disease (*SLC11A1 1730G>A* $P_{genotypic}$ = 0.0001, OR: 3.43, 95% CI: 1.95-5.93, *SLC11A1 469+14G>C* $P_{genotypic}$ = 0.006, OR: 15.91, 95% CI: 0.92-273.46). The involvement of SLC11A1 in the handling and elimination of intracellular pathogens, as well as its association with mycobacterial diseases makes it a biologically plausible candidate risk gene for CD. The results of recent genome-wide association studies strongly suggest defects in genes involved in bacterial detection, handling, and elimination are central to CD pathogenesis. Furthermore the assertion, albeit controversial, that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is an initial trigger for CD provides an additional rationale to investigate *SLC11A1* as a candidate risk gene for IBD. As a result, this study had two aims. The first was to attempt the first independent replication of the association of *SLC11A1 1730G>A* and *SLC11A1 469+14G>C* with IBD. The second aim was to use previously collected MAP IS900 data[20] to test for association of *SLC11A1* genotypes with occurrence of MAP DNA in peripheral blood.

MATERIALS AND METHODS

Study participants

Patients were selected from a New Zealand Caucasian IBD cohort that had been recruited to investigate genetic and environmental factors that contribute to CD and UC etiology[20-24]. Detailed phenotypic data were available for members of this cohort including ancestry, location of disease, family history of IBD, age of onset, presence of extra-intestinal manifestations, and requirement for surgery. The MAP status of the CD patients in this cohort had been determined previously using IS900 polymerase chain reaction[20]. Randomly selected blood donors (n = 501) from Christchurch (New Zealand), including 180 who had been previously tested for MAP status[20] served as controls.

Genotyping

Genotyping of *SLC11A1 1730G>A* (*rs17235409*) and *SLC11A1 469+14G>C* (*rs3731865*) was performed in 384-well plates using the pre-designed Taqman® SNP genotyping assays C_256352269_10 and C_1659793_10 (Applied Biosystems, Foster City, CA, USA) in a LightCycler® 480 Ⅱ (Hoffmann La Roche, Basel, Switzerland). Cycling conditions for *rs17235409* were 10 min at 95℃, 40 cycles of 15 s at 92℃ and 1 min at 60℃, and 30 s of cooling at 40℃. Conditions were the same for *rs3731865*, but annealing was at 66℃ rather than 60℃. Results were analyzed using Lightcycler® 480 software version 1.5.0. The accuracy of the genotyping assays was confirmed by repeat analysis of 13% of samples. Concordance between original and repeat genotype calls was 99%.

Statistical analysis

A web-based calculator (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl) was used to test for deviations from Hardy-Weinberg Equilibrium (HWE). The χ^2 and OR analyses were performed using SPSS for Windows, version 13.0 (SPSS Inc., Chicago, IL, USA). Associations were considered significant if P was < 0.05. *Post hoc* power analysis demonstrated that our cohort had 90% power to detect a relative risk of 2.15 for *SLC11A1 1730G>A* ($MAF_{controls}$ = 0.02, α = 0.05) and 99.8% power to detect a relative risk of 1.5 for *SLC11A1 469+14G>C* ($MAF_{controls}$ = 0.30, α = 0.05).

Ethical considerations

All study participants provided written informed consent to be involved in ongoing IBD research, and ethical approval for this study was given by the Upper South Regional Ethics Committee (Canterbury, New Zealand).

RESULTS

Genotyping for *SLC11A1 1730A>G* and *469+14G>C* was successful in 1468 (94.7%) and 1432 (92.4%) of study participants, respectively. No deviations from HWE were detected in cases or controls for either SNP (P > 0.05). The percentage minor allele frequency (MAF) of *SLC11A1 1730G>A* and *SLC11A1 469+14G>C* in our controls was 2% and 30%, respectively. We found no evidence of association of either *SLC11A1* SNP with overall CD, UC or IBD susceptibility (Table 1). Similarly, the minor allele and genotype frequencies of *SLC11A1*

Table 1 Genotype and allele frequencies of *SLC11A1 1730G>A* and *469+14G>C* in New Zealand Crohn's disease and ulcerative colitis patients, and healthy controls *n* (%)

Phenotype	Genotype			MAF	Allelic *P* value	Allelic OR (95% CI)
1730G>A	GG	GA	AA	A		
CD (*n* = 495)	474 (96)	21 (4)	0	21 (2)	0.832	1.07 (0.57-2.00)
UC (*n* = 470)	450 (96)	20 (4)	0	20 (2)	0.827	1.07 (0.57-2.02)
HC (*n* = 503)	483 (96)	20 (4)	0	20 (2)		
469+14G>C	GG	GC	CC	C		
CD (*n* = 495)	265 (54)	192 (39)	38 (8)	268 (27)	0.153	0.83 (0.65-1.07)
UC (*n* = 451)	245 (54)	171 (38)	35 (8)	241 (27)	0.101	0.81 (0.62-1.04)
HC (*n* = 486)	238 (49)	204 (42)	44 (9)	292 (30)		

MAF: Minor allele frequency; OR: Odds ratio; CI: Confidence interval; CD: Crohn's disease; UC: Ulcerative colitis; HC: Healthy controls.

Table 2 Genotype frequencies of *SLC11A1 1730G>A* (*rs17235409*) in inflammatory bowel disease patients who have used/not used immunomodulators *n* (%)

Phenotype/immunomodulator status	Genotype			*P* value	OR (95% CI)
	GG	GA	AA		
CD/never used IM (*n* = 217)	203 (94)	14 (6)	0	0.031	0.38 (0.15-0.95)
CD/have used IM (*n* = 278)	271 (98)	7 (2)	0		
UC/never used IM (*n* = 356)	336 (94)	20 (6)	0	0.010	0.75 (0.71-0.79)
UC/have used IM (*n* = 114)	114 (100)	0	0		
IBD/never used IM (*n* = 573)	539 (94)	34 (6)	0	0.002	0.29 (0.13-0.66)
IBD/have used IM (*n* = 392)	385 (98)	7 (2)	0		

OR: Odds ratio; CI: Confidence interval; CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; IM: Immunomodulator.

Table 3 Distribution of *SLC11A1 469+14G>C* genotype by *Mycobacterium avium* subspecies *paratuberculosis* status[1] in New Zealand Caucasians *n* (%)

MAP DNA in blood	Genotype frequency		*P* value	OR (95% CI)
	GG	GC + CC		
Present (*n* = 150)	66 (44)	84 (56)	0.02	1.56 (1.06-2.29)
Absent (*n* = 351)	193 (55)	158 (45)		

[1]Tested by IS900 polymerase chain reaction to detect the presence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) DNA in peripheral blood[20]. OR: Odds ratio; CI: Confidence interval.

1730G>A and *469+14G>C* did not associate with age of disease onset, disease behavior, disease location, or requirement for resectional surgery (all *P* values > 0.1, data not shown). A significantly higher frequency of the *SLC11A1 1730A* allele was seen in IBD patients who did not require immunomodulator therapy, compared to those who did require this treatment approach (P_{IBD} = 0.002, OR: 0.29, 95% CI: 0.13-0.66, P_{CD} = 0.03, OR: 0.38, 95% CI: 0.15-0.95, P_{UC} = 0.01, OR: 0.75, 95% CI: 0.71-0.79) (Table 2). There was no significant association of *SLC11A1 1730G>A* with MAP status, whereas the *SLC11A1 469+14C* allele was associated with increased incidence of MAP DNA in peripheral blood (*P* = 0.02, OR: 1.56, 95% CI: 1.06-2.23) in our cohort (Table 3).

DISCUSSION

Previous association of *SLC11A1 1730G>A* and *469+14G>C* with mycobacterial infections and preliminary evidence of association with CD[10-12,25] suggest that *SLC11A1* alters susceptibility to IBD. The primary aim of our study was to conduct the first independent replication of the association of *SLC11A1* with CD. In contrast to the original study of Gazouli *et al*[18], we found no evidence of *SLC11A1 1730G>A* or 469+14G>C as risk factors for IBD, CD or UC (all *P* values > 0.8) (Table 1). Comparison of the MAFs for the two *SLC11A1* SNPs revealed the existence of significant heterogeneity between Gazouli *et al*[18] and other studies for *SLC11A1 1730A*, and between populations of Northern versus Southern European ancestry for *SLC11A1 469+14C*. Our cohort and the cohort of Liu *et al*[26], which were composed primarily of individuals of Northern European ancestry, had *SLC11A1 469+14C* frequencies of 30% and 27% respectively. In contrast, the cohorts drawn from Southern European populations (Italian, Greek, and Turkish) exhibited significantly lower MAFs for this SNP. These differences in MAF distribution hint at the existence of a North-South gradient for *SLC11A1*, which could in turn explain the discordance between our study and that of Gazouli *et al*[18]. The occurrence of such gradients is not without precedence. The frequency of the CD-associated SNPs, *R702W*, *G908R* and *1007fs*, within the nucleotide oligomerization binding domain 2 gene (*NOD2*, also known as *CARD15*) exhibits a strong North-South gradient within Europe. A recent meta-analysis of *NOD2* association studies performed on European IBD cohorts has found that the MAFs and thus the contribution of these SNPs to CD risk increased significantly with decreasing latitude[27].

The minor allele of *SLC11A1 1730G>A* was found to be significantly over-represented in the subset of our IBD patients who had never used immunomodulators, and by inference had less severe disease (Table 2). However, we saw no association with other markers of disease severity in our cohort. Due to the very low minor allele frequency (no minor allele homozygotes were observed), this result requires replication in other large cohorts to rule out a type 1 error.

The second aim of this study was to test for association of *SLC11A1* with MAP. The MAP status of 321 CD patients and 180 controls has been determined previously[20]. Combining these patients and controls, we found no association between MAP status and *SLC11A1 1730G>A*, but did find an association with *SLC11A1 469+14G>C* (P = 0.02, OR: 1.56, 95% CI: 1.06-2.29) (Table 3). Earlier studies[14,16] on smaller CD cohorts (n = 37 or 59) did not find any evidence of association of MAP status with *SLC1A11 469+14G>C*. However, this polymorphism has been associated with susceptibility to *Mycobacterium tuberculosis*[10], and additional variation within *SLC11A1* has been associated with susceptibility to other mycobacterial diseases such as leprosy[9]. Our results provide preliminary evidence of an association of the *SLC11A1 469+14C* allele with susceptibility to MAP.

We conclude that although *SLC11A1* could be a risk factor for IBD in some Southern European populations, we did not find an association of *SLC11A1 469+14G>C* or *SLC11A1 1730G>A* with IBD in our cohort that comprised primarily patients of Northern European ancestry. However, the significantly higher incidence of MAP DNA in the peripheral blood of *SLC11A1 469+14C* heterozygotes and homozygotes compared to *SLC11A1 469+14G* within our cohort suggests that this *SLC11A1* SNP, although not directly influencing disease risk, might modify susceptibility to potential CD-causing bacteria.

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COMMENTS

Background

The involvement of *SLC11A1* in the handling and elimination of intracellular pathogens, as well as its association with mycobacterial diseases makes it a biologically plausible candidate risk gene for Crohn's disease (CD). The suggestion that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is an initial trigger for CD provides an additional rationale to investigate *SLC11A1* as a candidate risk gene for inflammatory bowel disease (IBD).

Research frontiers

A previous genetic association study has indicated that SLC11A1 is a susceptibility gene for IBD. The authors performed an independent replication of this study in a large population-based cohort of Northern European origin. They also tested for the association of these polymorphisms with MAP status.

Innovations and breakthroughs

This is believed to be the first study to examine the association of SLC11A1 polymorphisms in a well-powered cohort of Northern European origin. These findings indicate that *SLC11A1* polymorphisms do not modify disease risk for IBD, but might influence disease behavior (through indirect markers of severity) and susceptibility to MAP, a putative pathogen in CD. The authors also note the disparity of allele frequency between populations of Northern and Southern European origin.

Applications

By understanding how *SLC11A1* genotype influences the risk of colonization/infection with MAP, the authors might gain some insight into the contribution of this bacterium to IBD, and how defective clearance of MAP and other intracellular bacteria might be associated with modified disease risk.

Terminology

SLC11A1, solute carrier family 11 gene (also known as Natural Resistance Associated Macrophage Protein 1, *NRAMP1*) plays a key role in an effective innate immune response against intracellular pathogens. MAP is an intracellular bacterium that has been cited in several studies as a putative causal agent of CD.

Peer review

This paper provides interesting new results regarding the possible relationship between SLC11A1 polymorphisms and IBD risk. The study has been done carefully and thoroughly, and the paper is very well written. The lack of association of SLC11A1 and IBD risk in the study population (New Zealand Caucasians primarily of Northern European descent) is an important finding. The positive result that shows an association of an SLC11A1 allele and MAP status is novel and interesting.

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BRIEF ARTICLE

Anti-pancreatic antibody in Turkish patients with inflammatory bowel disease and first-degree relatives

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Abstract

AIM: To identify the role of anti-pancreatic antibody (PAB) in the diagnosis of inflammatory bowel diseases (IBD) among Turkish patients, and its frequency in first-degree relatives.

METHODS: PAB and anti-Saccharomyces *cerevisiae* (ASCA) were examined in serum samples of 214 subjects including patients with Crohn's disease (CD, n = 64), ulcerative colitis (UC, n = 63), first-degree relatives of patients with CD (n = 25), first-degree relatives of patients with UC (n = 28),and a control group with gastrointestinal symptoms other than (IBD) (n = 34) by indirect immunofluorescence Positivity of PAB and ASCA was compared in terms of Vienna classification, disease activity and medications used.

RESULTS: In terms of PAB positivity, no difference was found between patients with CD (14.1%) and UC (7.9%) however, significant difference was observed between patients with CD and subjects in the control group (P < 0.05). No difference was found between patients with CD and their relatives in terms of ASCA positivity, whereas a significant difference was found between other groups (P < 0.001). Compared to ASCA, the sensitivity of the PAB was 19% (7/37), its specificity was 93% (25/27), positive predictive value was 77% (7/9) and negative predictive value was 45% (25/55). ASCA was found with significantly higher prevalence in patients with CD activity index > 150 (P < 0.05).

CONCLUSION: PAB is valuable in the diagnosis of IBD rather than CD, but cannot be used alone for diagnostic purposes. PAB is not superior to ASCA in CD diagnosis and in detecting CD among relatives of patients with CD.

Key words: Anti-pancreatic antibody; Anti-Saccharomyces *cerevisiae* antibody; Crohn's disease; Ulcerative colitis; Inflammatory bowel disease

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INTRODUCTION

The incidence of Crohn's disease (CD) and ulcerative colitis (UC) is gradually increasing. Despite clinical, endoscopic, radiological andhistopathological findings, about 10% of patients with CD and UC are misclassified[1]. Moreover, 10% of the cases are not classified and referred to as indeterminate colitis. New treatment options for inflammatory bowel disease (IBD) are available today. Medical treatment and surgical operations to be implemented vary depending on the type of the disease.

Several serological indicators can be used for differential diagnosis[2]. The most commonly used two indicators are anti-Saccharomyces *cerevisiae* (ASCA) and perinuclear anti-neutrophil cytoplasmic antibodies (pANCA)[3]. Anti-pancreatic antibody (PAB) is another indicator that is currently under investigation in this regard. Serological indicators are not sensitive enough in IBD screening. Therefore, depending on merely serological indicators, diagnosis and treatmentof IBD are not possible. Several studies that areexamining the serological indicators in diagnosis and treatment are underway.

The familial occurrence of IBD is well known. Around 5.5%-22.5% of patients with IBD have another family member also affected with the disease[1-3]. In fact, the most important risk factor for IBD is having a family member with the disease. The relative risk for a sibling of a CD patient to also become affected is 13-36, and for a sibling of a UC patient this risk is 7-17[4], therefore first-degree relatives with no complaints are at risk of developing the disease. Early diagnosis is considered to decrease the disease complications, as well as possible surgical treatment in the long term. Although the search for predictive markers that could identify family members at risk for IBD has been intensive, no such markers have been identified to date. Studies with auto antibodies are being carried out, as these are non-invasive assays. Positive serum antibody findings in first-degree relatives of patients with IBD will, in most cases, indicate potential disease.

To the best of our knowledge, there are no studies that are investigating PAB in CD in Turkey. In our study, PAB was compared with ASCA, which is the most commonly used serological indicator in CD. The five groups included in the study were: patients with CD, patients with UC, first-degree relatives of patients with CD, first-degree relatives of patients with UC, and control subjects. The study aimed to: identify whether PAB plays a role in the differential diagnosis of CD; compare PAB with ASCA; determine the frequency of PAB in first-degree relatives of CD patients who carry potential risk for the disease; and determine whether PAB can contribute to early diagnosis in first-degree relatives.

MATERIALS AND METHODS

Patient enrollment

Among outpatients and inpatients who presented to Sisli Etfal Education and Research Hospital Department of Gastroenterology; we enrolled 64 patients with CD, 63 with UC, 25 first-degree relatives of patients with CD, 28 first-degree relatives of patients with UC, and 34 control patients with gastrointestinal symptoms other than IBD. Diagnosis of CD and UC was established by means of clinical, endoscopic and histopathological examinations. Exclusion criteria were; infective enterocolitis (excluded *via* feces microscopy, culture, serological examination for bacterial and ameba infection, staining of the biopsy with acid-resistant dye and bacterial culture), Behcet's disease, microscopic colitis and indeterminate colitis. Patients with a diagnosis of IBD for > 6 mo were enrolled. Enrolled patients were categorized according to age, sex, disease type, disease activation, clinical picture and involvement site of the disease.

Patients with UC were classified with proctitis, distal involved colitis, left colon involved colitis, diffuse colitis, and pancolitis. First-degree relatives involved either the siblings or the parents of the patients with IBD. First-degree relatives who had suspected complaints and histories of IBD were excluded. The control group consisted of patients from outpatient clinics who presented with gastrointestinal symptoms other than IBD, and who had no first-degree relative with IBD. Table 1 summarizes the demographical characteristics, disease duration, disease type and sites involvement, and medication being taken for all the enrolled groups.

For the activity of the diseases, CD activity index (CDAI) for CD and Trulove and Witts clinical activity index for UC were used. Medication was classified into seven groups: no medication; 5-aminosalicylic acid (ASA); azathioprine (AZA); 5-ASA + corticosteroid (CS); 5-ASA + AZA; 5-ASA + antibiotics and 5-ASA + CS + AZA.

Conduction of antibody testing

All tests were conducted by product specialists from Euroimmun AG, Turkey, who were trained at Euroimmun Laboratories in Germany, and were confirmed by a second specialist. Both specialists were blinded to the diagnoses. After being collected from patients, relatives and control group, venous blood samples were centrifuged within 3 h, and serum was separated and were maintained at 80℃ until the time of testing. Serum samples to be studied were transported to laboratories in ice boxes.

Determination of PAB

The presence of PAB was determined by indirect immunofluorescence staining in primate pancreas tissue (Figure 1). Kits prepared by Euroimmun AG were used. Substrates that were developed from pancreatic tissue from primates were divided into thin sections (biochips), transferred to slides, and utilized in the kit. The materials in the kits were kept under suitable conditions (4-8℃) until use For dilution and washing steps, we used a solution that was prepared from 10.2 g phosphate buffer, pH 7.2 and 2 mL Tween 20 (organic detergent). Antibodies tend to precipitate after the serum is dissolved, therefore serum samples of each patient were subjected to a string step (vortexing) to ensure homogeneous dispersion in the serum. Dilutions of 1/10 with PBS were prepared for

Table 1 Characteristics of patients, relatives and control group subjects *n* (%)

	CD (*n* = 64)	CD relatives (*n* = 25)	UC (*n* = 63)	UC relatives (*n* = 28)	Control (*n* = 34)
Sex					
Male	28 (43.8)	9 (36.0)	32 (50.8)	11 (39.3)	15 (44.1)
Female	36 (56.2)	16 (64.0)	31 (49.2)	17 (60.7)	19 (55.9)
Age (yr, mean ± SD)	37.93 ± 14.01	32.12 ± 14.31	38.74 ± 13.13	35.35 ± 17.07	38.58 ± 15.09
Disease duration (yr, mean ± SD)	4.37 ± 3.61		4.03 ± 3.66		
Disease location (CD)					
Colon	2 (3.1)				
Ileum	26 (40.6)				
Ileocolonic	36 (56.3)				
Disease location (UC)					
Proctitis			9 (14.3)		
Distal involvement			24 (38.1)		
Left colon invol			9 (14.3)		
Diffuse			2 (3.2)		
Pancolitis			19 (30.2)		
Disease type (CD)					
Stricturing	7 (10.9)				
Penetrating	6 (9.4)				
Inflammatory	51 (79.7)				
Medications (for CD and UC)					
No medication	5 (7.8)		5 (7.9)		
5-ASA	57 (89.06)		57 (90.4)		
Steroids	5 (7.8)		5 (7.9)		
AZA	13 (20.3)		8 (12.6)		
Antibiotics	1 (1.6)		3 (4.7)		

CD: Crohn's disease; UC: Ulcerative colitis; 5-ASA: 5-aminosalicylic acid; AZA: Azathioprine.

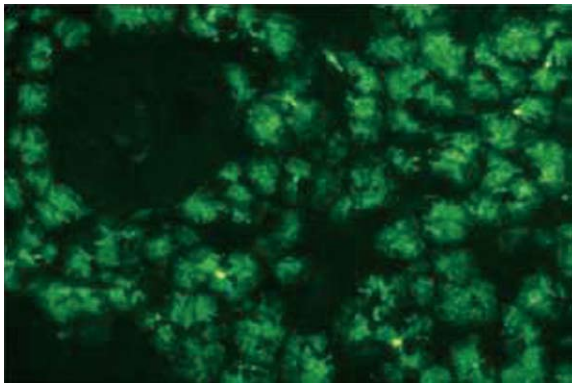

Figure 1 Pancreatic antibodys were determined by indirect immunofluorescence staining in primate pancreas tissue.

each patient. Prepared dilutions were incubated at room temperature (18-25℃) for 30 min, followed by 5 min washing with PBS. Later, incubation with fluorescence-marked anti-human globulin IgA and IgG conjugates was performed for each patient. After being washed with PBS for 5 min, the slides were prepared for examinations by specialists by adding glycerol, pH 8.4, included in the kit and closing with lamella.

Determination of ASCA

Kits prepared by Euroimmun AG that contained antibodies against S. *cerevisiae* were used. Storage conditions of this kit were the same as for the PAB kit. The same solutions were used for dilutions and washing steps. However, we used 1/100 dilutions for ASCA rather than 1/10 as in the previous preparation. Dilutions were incubated at room temperature (18-25℃) for 30 min, followed by 5 min washing with PBS. Later, incubation with fluorescence-marked anti-human globulin IgA and IgG conjugates was performed for each patient. After washing with PBS for 5 min, the slides were prepared for examination by specialists by adding glycerol, pH 8.4, included in the kit, and closing with lamella.

Statistical analysis

Data were evaluated using (SPSS) for Windows, version 9.05 statistical software. Research findings were converted into numeric and percentage distributions and Fischer's exact χ^2 test (used when the number of the object was < 20, or between 20 and 40 but if the least expectant value was < 5) and Pearson's χ^2 test were used to determine the level of significance of correlations between dependent (PAB and ASCA), independent (disease duration, disease onset age, disease type, site of involvement, medication) variables. $P < 0.05$ was considered statistically significant.

RESULTS

Demographic features of the involved patients are summarized in Table 1.

PAB was found to be positive in nine patients with CD (9/64; 14.06%) and five with UC (5/63; 7.93%) (Table 2, Figure 2), but the difference was not statistically significant.

ASCA was found to be positive in 57.80% (37/64) of patients with CD, and 20.60% (13/63) of patients with UC, 36% (9/25) of CD relatives, 17.90% (5/28) of UC

Table 2 Pancreatic antibody and anti-Saccharomyces *cerevisiae* positivity results in study groups *n* (%)

	CD (*n* = 64)	UC (*n* = 63)	CD relatives (*n* = 25)	UC relatives (*n* = 28)	Control (*n* = 34)
PAB	9 (14.1)	5 (7.9)	0 (0)	0 (0)	0 (0)
ASCA	37 (57.8)	13 (20.6)	9 (36)	5 (17.9)	6 (17.6)

CD: Crohn's disease; UC: Ulcerative colitis; PAB: Anti-pancreatic antibody; ASCA: Anti-Saccharomyces *cerevisiae*.

Table 3 Positivity rates of pancreatic antibody according to Vienna classification

		A1 (*n* = 43, PAB = 7, 16.27%)				A2 (*n* = 21, PAB = 2, 9.52%)		
		B1 (*n* = 31, PAB = 5, 16.12%)	B2 (*n* = 6, PAB = 1, 16.66%)	B3 (*n* = 6, PAB = 1, 16.66%)		B1 (*n* = 20, PAB = 2, 10%)	B2 (*n* = 1, PAB = 0, 0%)	B3 (*n* = 0, PAB = 0, 0%)
L1 (*n* = 26, 3.84%)	A1 (*n* = 18, PAB = 1, 5.55%)	*n* = 15, PAB = 1, 6.66%	*n* = 0, PAB = 0, 0%	*n* = 3, PAB = 0, 0%	A2 (*n* = 8, PAB = 0, 0%)	*n* = 7, PAB = 0, 0%	*n* = 1, PAB = 0, 0%	*n* = 0, PAB = 0, 0%
L2 (*n* = 2, 0%)	A1 (*n* = 1, PAB = 0, 0%)	*n* = 1, PAB = 0, 0%	*n* = 0, PAB = 0, 0%	*n* = 0, PAB = 0, 0%	A2 (*n* = 1, PAB = 0, 0%)	*n* = 1, PAB = 0, 0%	*n* = 0, PAB = 0, 0%	*n* = 0, PAB = 0, 0%
L3 (*n* = 36, 22.22%)	A1 (*n* = 24, PAB = 6, 25%)	*n* = 15, PAB = 4, 25%	*n* = 6, PAB = 1, 16.66%	*n* = 3, PAB = 1, 33.33%	A2 (*n* = 12, PAB = 2, 16.66%)	*n* = 12, PAB = 2, 16.66%	*n* = 0, PAB = 0, 0%	*n* = 0, PAB = 0, 0%

A1: Disease onset age < 40 yr; A2: Disease onset age ≥ 40 yr; B1: Inflammatory type; B2: Stricturizing type; B3: Fistulizing type; L1: Ileal involvement; L2: Colonic involvement; L3: Ileocolonic involvement. PAB: Pancreatic antibody.

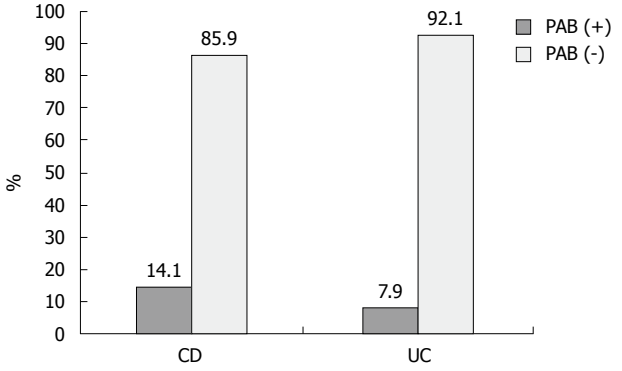

Figure 2 Pancreatic antibody frequency in Crohn's disease and ulcerative colitis patients. CD: Crohn's disease; UC: Ulcerative colitis; PAB: Pancreatic antibody.

relatives, and 17.60% (6/34) of the control subjects. A statistically significant difference was found between the patients with CD and UC and between the relatives of patients with UC and the control group ($P < 0.001$ for both), but there was no significant difference between the patients with CD and their relatives.

Sensitivity and specificity of PAB was 19% (7/37) and 93% (25/27), respectively. Positive predictive value was 77% (7/9) and negative predictive value was 45% (25/55). The likelihood ratio was 2.7 (0.19/10.93) and coherence was 0.50 (7+25/64).

Although frequencies of PAB and ASCA were found to be higher among the patients aged < 0 years than in the older group (Tables 3 and 4), the differences were not statistically significant for PAB or ASCA. The positivity ratios of ASCA and PAB were not significantly different between the patients with UC and CD.

When CD activity was classified as mild and severe, no difference was found in PAB positivity (Table 5). The same assessment performed for ASCA yielded no statistically significant difference.

CD patients were grouped according to medication and classified according to PAB and ASCA positivity. No significant difference was found in patients using corticosteroids or immunosuppressive drugs in terms of PAB and ASCA levels.

DISCUSSION

Tissue damage in IBD is caused by multiple mechanisms that are mediated by the immune system. Although several studies have supported the argument that autoimmune mechanisms are the primary responsible mechanism, the consensus is that autoimmune stimulation does not have direct significance. Therefore, IBD is not actually an autoimmune disease. However, because the pathogenesis is mediated by the immune system, determination of autoantibodies and other immunological indicators is of clinical importance and warrants further research[5].

PAB frequency in patients with CD was found as 14.06% (9/64) and as 7.93% (5/63) for UC patients in the present study. No PAB positivity was noted in the relatives of both CD and UC patients. Although PAB was more positive in CD patients, the difference was not statistically significant. Related studies in the literature have reported PAB frequencies of 15%-40% in CD, 1%-4% in UC, and 1%-4% in control subjects[6-15]. The results of the present study can be considered consistent with those of Greek

Table 4 Positivity rates of anti-Saccharomyces *cerevisiae* according to Vienna classification

		A1 (*n* = 43, ASCA = 27, 62.79%)				A2 (*n* = 21, ASCA = 10, 47.61%)		
		B1 (*n* = 31, ASCA = 18, 58.06%)	B2 (*n* = 6, ASCA = 3, 50%)	B3 (*n* = 6, ASCA = 6, 100%)		B1 (*n* = 20, ASCA = 9, 45%)	B2 (*n* = 1, ASCA = 1, 100%)	B3 (*n* = 0, ASCA = 0, 0%)
L1 (18/26, 69.23%)	A1 (*n* = 18, ASCA = 13, 72.22%)	*n* = 15, ASCA = 10, 66.66%	*n* = 0, ASCA = 0, 0%	*n* = 3, ASCA = 3, 100%	A2 (*n* = 8, ASCA = 5, 62.5%)	*n* = 7, ASCA = 4, 57.14%	*n* = 1, ASCA = 1, 100%	*n* = 0, ASCA = 0, 0%
L2 (0/2, 0%)	A1 (*n* = 1, ASCA = 0, 0%)	*n* = 1, ASCA = 0, 0%	*n* = 0, ASCA = 0, 0%	*n* = 0, ASCA = 0, 0%	A2 (*n* = 1, ASCA = 0, 0%)	*n* = 1, ASCA = 0, 0%	*n* = 0, ASCA = 0, 0%	*n* = 0, ASCA = 0, 0%
L3 (19/36, 52.77%)	A1 (*n* = 24, ASCA = 14, 58.33%)	*n* = 15, ASCA = 8, 53.33%	*n* = 6, ASCA = 3, 50%	*n* = 3, ASCA = 3, 100%	A2 (*n* = 12, ASCA = 5, 41.66%)	*n* = 12, ASCA = 5, 41.66%	*n* = 0, ASCA = 0, 0%	*n* = 0, ASCA = 0, 0%

A1: Disease onset age < 40 yr; A2: Disease onset age ≥ 40 yr; B1: Inflammatory type; B2: Stricturizing type; B3: Fistulizing type; L1: Ileal involvement; L2: Colonic involvement; L3: Ileocolonic involvement. ASCA: Anti-Saccharomyces *cerevisiae*.

Table 5 Pancreatic antibody and anti-Saccharomyces *cerevisiae* positivity according to Crohn's disease activity index *n* (%)

Disease activity according to CDAI	PAB		ASCA		Total
	Negative	Positive	Negative	Positive	
Mild (< 150 points)	49 (84.5)	9 (15.5)	27 (46.6)	31 (53.4)	58 (100)
Moderate (150-450 points)	5 (100)	0 (0)	0 (0)	5 (100)	5 (100)
Severe (> 450 points)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)
Total	55	9	27	37	64

CDAI: Crohn's disease activity index; PAB: Pancreatic antibody; ASCA: Anti-Saccharomyces *cerevisiae*.

and Belgian studies that have demonstrated that PAB is present not only in patients with CD, but also in those with UC. Frequency of antibody in patients with UC was reported to be 23.3% in a Belgian study and 24.7% in a Greek study[11-14]. As PAB positivity is also seen in patients with UC, and because the frequency in patients with UC is not significantly different from that in CD patients, PAB seems to be a more suitable indicator for IBD rather than CD. In the light of these findings, PAB cannot be regarded as a screening test for IBD. Nishimori *et al*[13], Desplat-Jégo *et al*[15] and Koutroubakis *et al*[11] have arrived at the same conclusion.

Several serological indicators that are useful in differential diagnosis of UC and CD have been studied recently, and utilization of multiple indicators has been shown to improve differential diagnosis in cases of indeterminate colitis. Some of these indicators are pANCA, ASCA, PAB, OmpC antibody and I-2 and anaerobic coccoid rods antibodies[8-19]. This is why ASCA, together with PAB, was examined in the present study. As shown in Table 2, ASCA positivity was 57.8% (37/64) in CD patients, 20.6% (13/63) in UC patients, 36% (9/25) in the first-degree relatives of patients with CD, 17.9% (5/28) in the first-degree relatives of patients with UC, and 17.6% (6/34) in the control group. These values are much higher than the PAB positivity rates. In terms of these positivity rates, a statistically significant difference was identified between CD and UC, and relatives of UC patients and control subjects ($P < 0.001$); however, no significant difference was noted between CD patients and their relatives between UC patients and their relatives,or between UC patients and the control group. PAB seems to be a less desirable serological indicator compared to pANCA and ASCA in patients with IBD, as our findings indicate a lower prevalence. However, it can improve diagnosis and the predictive value when used in combination with common indicators.

ASCA, which is found with higher rates of positivity in patients with CD, is reported to be positive also in spondyloarthropathy associated with HLA-B27, which is its disadvantage[8]. In contrast, studies of PAB, have emphasized that it has a high specificity for CD and is positive only in patients with CD[8,17,18].

In a study by Stöcker *et al*[9], higher rates of PAB positivity were noted in patients with disease duration > 2.5 years compared to those with duration < 2.5 years. Similarly, Klebl *et al*[16] have also identified a significant relationship between PAB positivity and disease duration ($P = 0.04$). Koutroubakis *et al*[11] have compared patients with disease duration less and more than 2 years, and have noted a tendency towards increased PAB positivity in the latter; however, the result was not statistically significant. When PAB frequency was assessed in terms of disease duration in our study, positive findings were identified for 14.3% (5/35) of the patients with disease duration < 3 years compared to 13.8% (4/29) in those with disease duration > 3 years. The results are very close to each other and no difference was detected with the statistical methods used in this study (Fisher's exact χ^2 test).

Another research topic in studies with PAB is the relationship of PAB with disease activity. Most of the earlier studies[7,11,19,20] have failed to identify a correlation between PAB positivity and disease activity, and a parallel was found only by Goischke *et al*[12]. There were no significant relationships between disease activity and PAB positivity in our study (Fisher's exact χ^2 test).

According to the disease type of CD, PAB was positive in 13.72% of patients with inflammatory type, in 14.28% of patients with stricturing type, and in 16.66% of patients with fistulizing type disease. The differences between the groups mentioned above were not found to be statistically significant, although a higher frequency was noted for the fistulizing type. Antibody frequency has been shown to be higher in fistulizing and stricturing type diseases[11,16]. Klebl *et al*[16] have found that PAB frequency was 31.5% in stricturing and non-penetrating type, 41.7% in stricturing type, and 31.5% in penetrating type disease. Koutroubakis *et al*[11], on the other hand, have reported the frequency as 60% in stenotic type, 28.6% in inflammatory type, and 41.2% in fistulizing type disease, with a statistically significant result for stenotic type compared to the other types.

The most problematic cases in differential diagnosis are CD patients with isolated colon involvement, as the sites of involvement are the same as with UC. Sites of involvement in CD patients in this study were ileum for 26, ileocolon for 36 and colon for two patients. There were very few subjects with colon involvement, therefore, it was not possible to perform a comparison with subjects with ileum involvement. PAB positivity of patients with ileocolon involvement was higher than for other groups, but without statistical significance. There were also no significant differences in ASCA results according to site of involvement. These results were in accordance with several other relevant studies. In their studies from 1991 and 1996, Seibold *et al* did not find a relationship between PAB and site of bowel involvement[7-21]. Klebl *et al*[16] noted no relationship between PAB positivity and L category by the Vienna classification system. Koutroubakis *et al*[11] did not detect a significant relationship between disease localization and PAB positivity, but found less frequent PAB positivity in CD with colon involvement as compared to ileum or ileocolon involvement ($P = 0.1$). Similarly, no relation between PAB and disease localization was identified by Lawrance *et al*[22].

Another issue investigated in PAB studies is the relationship between PAB and concomitant drugs. Despite the studies by Stöcker *et al*[9] and Folwaczny *et al*[10] that have reported a high antibody frequency in patients not using glucocorticosteroids, Seibold *et al*[7,21], Goischke *et al*[12] and Klebl *et al*[16] did not detect any relationship between PAB frequency and drug use, including glucocorticosteroids. In our study, PAB positivity rates showed no difference between CD patients who were and were not taking glucocorticosteroids. Similarly, no statistically significant difference was noted in patients who were and were not using AZA.

Of the patients with IBD in the present study, 5%-10% had a positive familial history. Farmer *et al*[23] observed that one third of IBD patients had family histories positive for IBD. It is known that the incidence of CD is 14-times higher in complaint-free first-degree relatives of CD patients compared to the general population, and that these individuals are at risk of developing the disease[24] whereas Monsén *et al*[25] found that the prevalence of CD among first degree relatives 21 times higher than among non-relatives. Some studies have indicated that PAB positivity has a low frequency in the first-degree relatives of patients with CD. Seibold *et al*[21] have investigated 606 patients and arrived at the conclusion that PAB was a specific indicator for CD, and that PAB was rarely positive in family members of CD patients (2.5%). They concluded that most of the PAB-positive family members were CD patients[5,19]. Folwaczny *et al*[10] have calculated PAB positivity in first-degree relatives of CD patients as 4%. The same study has reported that there were no significant differences between the incidence of PAB-positivity in the relatives of CD and patients and healthy controls[6]. On the other hand, the findings of Joossens *et al*[14] are inconsistent with those reported in the above studies; they have found PAB prevalence of 32% in CD, 23.3% in UC and 22.2% in family members of patients with IBD. One of the purposes of the present study was to investigate PAB and ASCA positivity in first-degree relatives of patients with IBD, and to determine whether these indicators would contribute to early detection of the disease in these risk groups. However, PAB was detected in none of the first-degree relatives of IBD patients, nor in any subjects in the control group. ASCA results for the relatives of patients with CD, relatives of UC patients and the control group did not differ significantly, either. Shanahan *et al*[19] had summarized that autoantibodies were unlikely to have a direct, primary pathogenic role in CD and viewed that autoantibodies as important bridge between clinical and basic science. Müller-Ladner *et al*[20] had also found a link between pancreatic antibodies and Crohn's disease. Therefore, we believe it would not be appropriate to use PAB or ASCA alone for determining the potential of CD patients' relatives to develop CD.

In conclusion, in our study based on the absence of a significant difference between positivity in CD and UC, PAB has a diagnostic value for detecting patients with IBD rather than CD, and it should not be used alone for diagnosis. Also, we can say that investigating PAB and ASCA in first-degree relatives of IBD patients does not offer much benefit in early detection of the disease, and there is no superiority of PAB over ASCA in clinical practice in terms of CD diagnosis and early detection of the disease in patients'relatives. Further studies with novel indicators are still needed in the diagnosis of CD, and early diagnosis in relatives of CD patients.

COMMENTS

Background

Several antibodies have been associated with inflammatory bowel disease (IBD), with one of the most comprehensively studied being antibodies against

anti-Saccharomyces *cerevisiae* (ASCA). Pancreatic antibodies (PABs) are also newly studied antibodies that are specific for Crohn's disease (CD) and ulcerative colitis (UC), but their sensitivity alone is low.

Research frontiers

PAB in combination with ASCA might increase the sensitivity for detecting CD, especially isolated colonic CD. This study focused on the value of PAB alone or in combination with ASCA for diagnosing IBD, and differentiating CD from UC.

Innovations and breakthroughs

Several serological indicators can be used for differential diagnosis of IBD. PAB is another indicator that is currently under investigation in this regard. Serological indicators are not sensitive enough for IBD screening. Therefore, depending on serological indicators alone, diagnosis and treatment of IBD will not be possible. Several studies that are examining serological indicators for diagnosis and treatment are underway. There are no studies that are investigating PAB in CD in Turkey. The present study aimed to: identify whether PAB plays a role in the diagnosis and differential diagnosis of CD; compare it with ASCA; determine its frequency in first-degree relatives of CD patients who carry a potential risk for the disease; and determine whether it can contribute to early diagnosis.

Applications

This study suggest that PAB is a novel indicator that is still needed in the diagnosis of CD, and early diagnosis in relatives of CD patients.

Peer review

This study aimed to identify the role of PAB in the diagnosis of CD, and determine its frequency in first-degree relatives. The main focus of this work was to measure the frequency of PAB and ASCA in Turkish IBD patients.

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BRIEF ARTICLE

Palliation of malignant esophageal obstruction and fistulas with self expandable metallic stents

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Abstract

AIM: To evaluate the efficacy of self expandable metallic stents (SEMS) in patients with malignant esophageal obstruction and fistulas.

METHODS: SEMS were implanted in the presence of fluoroscopic guidance in patients suffering from advanced and non-resectable esophageal, cardiac and invasive lung cancer between 2002 and 2009. All procedures were performed under conscious sedation. All patients had esophagus obstruction and/or fistula. In all patients who required reintervention, recurrence of dysphagia, hemorrhage, and fistula formation were indications for further endoscopy. Patients' files were scanned retrospectively and the obtained data were analyzed using SPSS 13.0 for Windows. The χ^2 test was used for categorical data and was analysis of variance for non-categorical data. Patients' long-term survival was assessed using the Kaplan-Meier method.

RESULTS: Stents were successfully implanted in 90 patients using fluoroscopic guidance. Reasons for stent implantation in these patients were esophageal stricture (77/90, 85.5%), external pressure (8/90, 8.8%) and tracheo-esophageal fistula (5/90, 5.5%). Dysphagia scores (mean ± SD) were 3.37 ± 0.52 before and 0.90 ± 0.43 after stent implantation (P = 0.002). Intermittent, non-massive hemorrhage due to the erosion caused by the distal end of the stent in the stomach occurred in only one patient who received implementation at cardio-esophageal junction. Mean survival following stenting was 134.14 d (95% confidence interval: 94.06-174.21).

CONCLUSION: SEMS placement is safe and effective in the palliation of dysphagia in selected patients with malignant esophageal strictures.

Key words: Esophagus cancer; Stenosis; Stents; Complication; Dysphagia

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INTRODUCTION

Patients with esophageal cancer and cancer of the gastro-esophageal junction commonly present with an advanced disease mainly because the esophagus is quite distendable and patients may not experience dysphagia until almost half of the luminal diameter is compromised[1]. At the time of diagnosis, a high percentage of patients with esophageal cancer have an advanced stage of disease and when the tumor is not operable, only palliative treatment is applicable, primarily to manage dysphagia[2]. Dysphagia is the most devastating symptom in malign stricture of the

esophagus. Aspiration is another frequent symptom requiring palliative therapy. This is either aspiration of saliva or food as a result of complete dysphagia or a tracheo-esophageal fistula. Similar problems may occur due to local pressure, particularly in patients with advanced lung cancer or mediastinal tumors. Early palliation of dysphagia and other symptoms is important in terms of nutritional status and a good quality of life. Among palliative treatments, radiotherapy, laser therapy and conventional plastic endoprostheses have a limited effect in preventing rapid weight loss due to malnutrition[3].

Several self expandable metallic stents (SEMS) are now available and have been used widely to provide immediate symptomatic relief of malignant dysphagia. They are useful for patients with poor functional status who cannot tolerate radiotherapy or chemotherapy, who have advanced metastatic disease, or in whom previous therapy has failed[3]. The aim of this report was to summarize our experience with expandable metal stents for palliation of malignant dysphagia in our 90 patients.

MATERIALS AND METHODS

From September 2002 to December 2009, 90 patients (65 men, 25 women; mean age 61.57 years, range 38-85 years) with malignant inoperable esophageal obstruction and high grade dysphagia or fistula were treated using flexible self-expanding metallic stents. Surgery was considered to be contraindicated in all patients due to the patients' poor general condition, the advanced stage of tumors, untreatable tumor recurrence or distant metastases. The ability to swallow was expressed as a dysphagia score. The scoring system was modified from that reported by Mellow and Pinkas; a score of 0 denoted the ability to eat a normal diet; 1, the ability to eat some solid food; 2, the ability to eat semisolid only; 3, the ability to swallow liquids only; and 4, complete dysphagia[4]. The data were compared with that published in the literature. All patients gave their informed consent for the procedure.

Stent material

We employed self-expendable covered metallic esophageal stents from different companies (Boston Scientific, Watertown, MA, USA and Micro-Tech, Nanjing, China) in 76 patients. A covered self expanding stent was used in 5 patients with esophagotracheal fistulas. Diameters and length of covered stents varied between 18 and 20 mm, and 6 and 15 cm, respectively. Uncovered metallic stents (Ultraflex Boston Scientific, Watertown, MA, USA) were used in 14 patients. Diameters and length of uncovered stents varied between 18 and 23 mm, and 10 and 15 cm, respectively.

Implantation procedure

Before stent implantation, patients were prepared by administering conscious sedation analgesia (2.5-5 mg midazolam and 25-50 mg meperidine iv) The entire length of tumor stenosis and the location of the esophagotracheal fistula, if available, were precisely determined by an endoscope with an 11 mm diameter. High-grade strictures that could not pass an endoscope were opened prior to stenting to a diameter of at least 8-10 mm using a balloon dilator. After the preparatory treatment, the upper gastrointestinal tract was inspected endoscopically and the tumor margins on both sides were marked on the patients' skin with metallic markers. After the placement of a guidewire into the stomach and the removal of the endoscope, the stent introducer was inserted over the guidewire into the esophagus under fluoroscopic guidance. The stent was positioned under fluoroscopic control with guidance of radio-opaque skin markers and then released from the delivery system. The stents expanded by themselves within 10-60 s. After another minute, the delivery system and the guidewire were carefully removed. After retraction of the delivery system, an immediate endoscopic check was made (Figure 1). A plain chest radiograph and a contrast study using swallowed water-soluble contrast medium were performed to ensure the correct positioning and expansion of the stent and to exclude perforation. In cases of esophago-tracheal fistula, a radiographic control using a water-soluble contrast medium was carried out (Figure 2). These patients were advised to consume only liquids until the stent position was checked. Then they had semisolid or solid food, as individually tolerated. Most patients were discharged on an outpatient basis and were instructed to

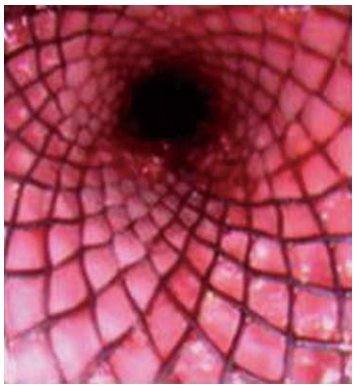

Figure 1 Proximal view of a fully opened stent.

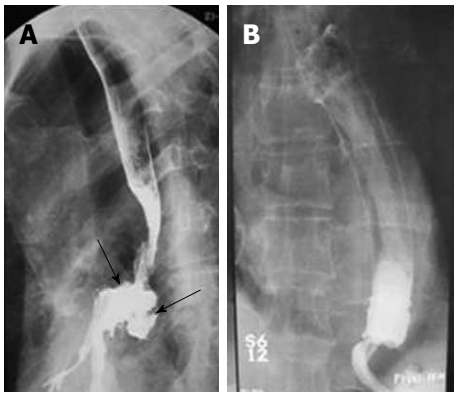

Figure 2 Palliation of tracheoesophageal fistula with stenting. A: Barium esophagogram showing a tracheo-esophageal fistula resulting from lung cancer; B: Complete occlusion of the fistula after stenting. Arrows show a large tracheoesophageal fistula.

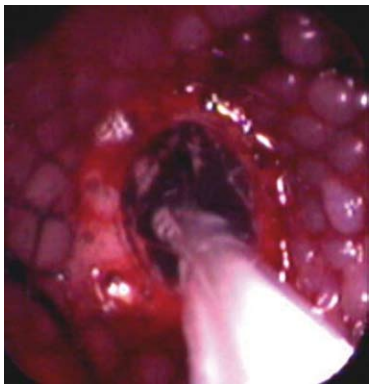

Figure 3 In some cases with insufficient stent expansion due to tight stricture, endoscopic balloon dilatation was performed through the opened stent.

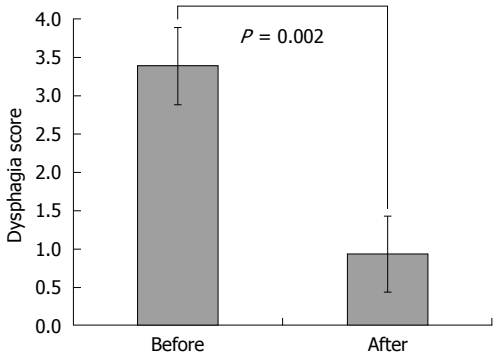

Figure 4 Comparison of oral alimentation status before and after placement of self expandable metallic stents. Figure shows the change in dysphagia score on day 3 after stenting. For the scoring system, see Materials and Methods section.

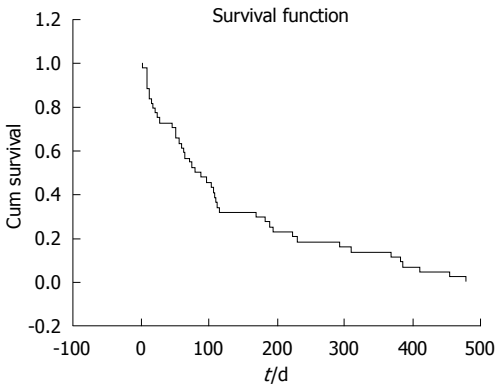

Figure 5 Kaplan-Meier survival curve of 90 patients following stenting.

start oral ingestion the same day. Anti reflux measures (proton pomp inhibitors and/or prokinetic agents) were administered to patients whose prosthesis extended beyond the gastroesophageal junction. We did not use anti-reflux stents.

In some cases, because of the kinking of the esophageal lumen due to malign stricture, insertion of the stent introducer was difficult. In such cases, an endoscope was inserted after the introducer to help push it through the narrowed malign segment. In 3 cases with distal esophageal polypoid tumors, debulking was performed with a polypectomy snare because of extensive vegetation in the lumen preventing stent positioning and expansion. In 17 cases with insufficient stent expansion due to tight stricture, an endoscopic balloon dilatation through the opened stent was performed immediately after stenting to obtain an appropriate passageway (Figure 3).

An exploratory analysis was performed by using SPSS 13.0. Analysis of variance was used for non-categorical (continuous) data and the χ^2 test was used for categorical data. Survival data were assessed by the Kaplan-Meier method.

RESULTS

A total of 100 expandable metal stents were placed in 90 patients for malignant dysphagia caused by esophageal cancer or extrinsic compression. Fifteen uncovered SEMS were placed in 14 patients and 85 covered stents were placed in 76 patients. Our series consisted of 25 women and 65 men with a mean ± SD age of 61.57 ± 12.05 years (range, 38-85 years). The mean size of the strictured segment of the esophagus where the stent was implanted was 7.14 ± 2.67 cm. The primary reason for stenting was esophageal stenosis alone in 77 patients (85.5%), followed by esophageal extrinsic compression in 8 patients (8.8%) and trachea-esophageal fistula in 5 patients (5.5%). In one patient stenting was performed in both his esophagus and the bronchus. In 4 cases, when the stent was opened in an inappropriate position, the stent was removed by pulling the attached string and a new stent was reinserted. Endoscopic balloon dilatation was performed before stenting in 27 patients (30%). Almost all patients improved in terms of oral intake. After the procedure, 84.4% of the patients (76/90) did not report any dysphagia during follow-up. Before stent placement, mean dysphagia score was 3.37 ± 0.52; after stent placement, mean dysphagia score was 0.90 ± 0.43 ($P = 0.002$) (Figure 4). There were no clinically significant complications during the insertion of stents. With respect to complications associated with stents, migration was noted in 4 patients (5%). Intermittent, non-massive hemorrhage due to the erosion caused by the distal end of the stent in the proximal stomach occurred in one patient who had received stent implantation in the cardio-esophageal junction. Migration was noted after 140 d on average (after 419 d in the first patient, after 69 d in the second patient, after 45 d in the third patient and after 27 d in the fourth patient). Migrations occurred following chemotherapy in 3 of the patients. Proximal tumor overgrowth was observed after 165 d on average following stenting in 6 patients (8.1%). Tumor overgrowth was observed within the first month following stenting only in one patient (at day 13). A second extendable stent was implanted in all of these patients. Minimal tissue ingrowth was detected in 3 patients (3.3%) treated with the uncovered stent and none had overt dysphagia.

Mean survival following stenting was 134.14 d [95% confidence interval: 20.45 (94.06-174.21)] (Figure 5). Restenting was needed in 10 patients (Table 1). No patient

Table 1 Characteristics of restented patients

Age (yr)	Sex	Reason for restenting	Tumor location (stage)	Stenosis location	Dilatation	Stent type
62	M	Proximal overgrowth	Gastric (T4)	Distal	No	6 cm, 20 mm CVRD
52	M	Proximal overgrowth	Lung Tm	Middle	No	6 cm, 20 mm CVRD
54	M	Proximal overgrowth	Gastric (T4)	Distal	APC	6 cm, 20 mm CVRD
71	M	Proximal overgrowth	Esophagus (T4)	Distal	No	6 cm, 20 mm CVRD
75	M	Migration	Lung Tm	Middle	No	10 cm, 20 mm CVRD
53	M	Proximal overgrowth	Esophagus (T4)	Proximal	No	6 cm, 18 mm CVRD
64	M	Migration	Esophagus (T3)	Middle	No	10 cm, 18 mm CVRD
67	M	Migration	Esophagus (T4)	Distal	No	10 cm, 20 mm UCVRD
55	F	Proximal overgrowth	Gastric (T4)	Distal	No	15 cm, 20 mm CVRD
49	F	Migration	Esophagus (T4)	Distal	No	6 cm, 20 mm CVRD

CVRD: Covered; UCVRD: Uncovered; APC: Argon plasma coagulation.

Table 2 Patient demographics

	n (%)
Total No. of patients	90
Mean age (yr, range)	61.57 (38-85)
Male/female	65/25 (72.2/27.8)
Present illness	
Esophagus carcinoma	54 (60.0)
Squamous cell carcinoma	47 (52.2)
Adenocarcinoma	7 (7.7)
Gastric carcinoma (and cardiac)	20 (22.2)
Lung carcinoma	16 (17.7)
Location of obstruction	
Proximal	5 (5.6)
Middle	31 (34.4)
Distal	54 (60.0)
Restenting	10 (11.1)

had esophageal perforation or procedure-related death. Dilatation was performed in 27 patients pre-operatively *via* a 12-16 mm balloon dilator for high grade strictures. Argon plasma coagulation was performed for one patient because of proximal tumor overgrowth.

Table 2 illustrates the localizations of the stents, the reasons for stenting and the patients' demographic data.

DISCUSSION

Our results suggest that SEMS provide a rapid and effective palliation for dysphagia in malignant stenosis, and low morbidity is associated with the procedure. In our study, all patients had significant relief of dysphagia. The frequency of the common conditions associated with stenting as identified in our study are presented in Table 3, in comparison with data reported from other studies[5-15].

Palliation is often difficult to achieve in patients with esophageal obstruction as a result of cancer. Among many endoscopic and nonendoscopic treatment alternatives for palliation of cancer-related dysphagia, stenting with SEMS is one of the main options. It is useful for patients with poor functional status who cannot tolerate radiation or chemotherapy, who have advanced metastatic disease, or in whom previous therapy has failed[16]. It can be concluded that stents provide better oral intake and quality of life compared to surgical palliation techniques. Despite the substantially higher cost of expandable metal stents as compared to traditional rigid plastic esophageal stents, there are substantial overall cost savings resulting from the reduction in the number of days of hospitalization for surgery and possible complications[7]. The majority of our subjects were patients who were referred to our clinic for dysphagia palliation from different centers and none were hospitalized after stenting. Our experience supported that SEMS could be inserted in outpatients, reducing the cost of treatment.

It has been shown that dysphagia was relieved in approximately 90% of patients who received an expendable metal stent[7,12,17]. Compared to other palliative methods, the most significant and fastest improvement in swallowing is achieved in patients undergoing implantation of SEMS[6,14,18,19]. Improvements in dysphagia were achieved in almost all of our patients following stenting. While the mean dysphagia score was 3.37 before stenting, the score was 0.90 following SEMS (P = 0.002). This decrease in dysphagia score is consistent with the literature. Recurrent dysphagia due to device migration and proximal overgrowth occurred in 10/90 (11.1%) of those who received SEMS. No significant difference was found between mean decreases in dysphagia scores in patients who were implanted with covered and uncovered stents (mean decreases in dysphagia scores in the 2 groups were 2.48 ± 0.53, 2.42 ± 0.51, respectively; P = 0.487). Covered and uncovered stents are equally good for dysphagia palliation[20].

Esophageal expandable metal stents are also used to treat tracheo-esophageal fistulas due to cancer. Tracheo-esophageal fistulas develop in patients with advanced esophageal and lung cancer and lead to devastating symptoms as a result of continuous aspiration of saliva and food. Survival over 30 d is rare in these patients, unless they undergo an occluding procedure using an endoprosthesis[18,21-25]. Noncovered stents are not suitable for the treatment of fistulas, because the esophageal contents can pass easily through the mesh and into the esophageal defect. The covered types of metal stents are considered as the primary choice of treatment since treatment of fistula with SEMS improves survival[18,26,27]. In our study group, fistulas were successfully closed with covered stents in all patients with a fistula. All 5 of these patients had primary lung tumors.

Table 3 Outcome of published series of self-expandable metallic stent insertion: with or without fluoroscopy for self-expandable metallic stent insertion

Series	*n*	Dilation rate (%)	Stents uncovered	Perforation rate (%)	Migration rate (%)	Overgrowth rate (%)	Ingrowth rate (%)	Reintervention rate (%)	Median survival (d)
Present series	90	30.0	15/85	0	4.4	6.6	3.3	15.5	134
Wilkes *et al*[5]1	98	8	95/5	0	3.1	25.5	-	39.8	100
Adam *et al*[6]	42	100	45/55	0	19	2.3	9.5	36	53
Knyrim *et al*[7]2	21	0	100/0	0	0	9.5	14.2	3.8	167
Sarper *et al*[8]	41	100	19/81	4.9	2.4	2.4	2.4	-	94
Siersema *et al*[9]	100	7	0/100	6	13	10	0	49	109
Christie *et al*[10]	100	77	76/24	1	8.7	4	29.1	51	-
Wengrower *et al*[11]3	81	-	100/0	3.6	5.95	2.4	0	-	120
De Palma *et al*[12]2	19	0	100/0	0	0	0	10.5	-	198
Kozarek *et al*[13]	38	100	5/95	3	18.4	23.6	5.2	80	90
White *et al*[14]1	70	100	57/43	2.8	0	1.4	2.8	-	-
Maroju *et al*[15]1	30	-	9/91	0	3	7	10	-	161

[1]Stent was placed under direct visualization in the study; [2]Study of self-expandable metallic stent (SEMS) *vs* plastic esophageal prostheses treatment, but only data from SEMS arm was included; [3]Only esophacoil stents were used.

Stents were implanted in 5 of our patients because of proximal constriction within 4 cm. The Ultraflex type of covered stent was implanted in all these patients. Stent intolerability due to foreign body sensation, aspiration, perforation, proximal migration or pain was not observed in any of these patients. Verschuur *et al*[28] reported 44 patients had a malignant stricture within 4 cm of the upper esophageal sphincter. In this study, dysphagia improved in most patients, and the occurrence of complications and recurrent dysphagia was comparable to that in patients who underwent stent placement in the mid and distal esophagus. It has been reported that 5%-15% of patients had a foreign body sensation; however, in none of the patients was stent removal indicated[29]. There is a continuing debate about the advisability of SEMS placement for proximal esophageal cancer. If placed, it is frequently recommended that a distance of 2 cm below the upper esophageal sphincter should be maintained while placing a stent.

Intraprocedural complications of endoscopic stenting include those associated with sedation, aspiration, malpositioning of the stent, and esophageal perforation. Immediate postprocedural complications may include chest pain, bleeding, and tracheal compression, with resultant airway compromise and respiratory arrest. Late complications include distal or proximal stent migration, formation of an esophageal fistula, bleeding, perforation, and stent occlusion. Approximately 0.5%-2% of patients who undergo the procedure die as a direct result of placement of an expandable metal stent[30]. No patients died during the procedure in our patient group. Perforation of the esophageal wall is potentially related to the device itself and prior chemoradiotherapy[31]. This complication is presumably due to stent-induced pressure necrosis within devitalized esophageal tissue. The majority of stenting complications are reported in the literature to occur with plastic endoprostheses. In a study by Knyrim *et al*[7], 42 patients with metallic stent and plastic endoprosthesis were compared and the frequency of complications was reported to be less in patients who were implanted with metallic stents (0 and 9 patients, respectively). Two prospective, randomized, controlled trials have shown a significantly lower rate of procedural complications using expanding metal stents[7,12]. Perforation as a direct complication of stent placement was not seen in our patients. This result of perforation compares favorably with other studies[18,32-37]. In contrast to placement of conventional esophageal stents, placement of expendable metal stents do not require a large-bore bougienage, thereby minimizing the risk of perforation and facilitating the insertion procedure. However, in our series balloon dilatation was performed in 27 patients before stenting. The frequency of dilatation has been reported to be in the range of 0%-100% in the literature[7,12,13].

A tracheoesophageal (TE) fistula is one of the late complications that may occur after SEMS. The incidence of fistulas following stenting has been reported as 0%-10% in the literature. The type of the implanted stent was found to have no role in fistula development[6,18,38,39]. Fistulas should be treated with placement of additional, overlapping covered metallic stents. Retrospective analysis of our patients did not reveal any patient developing fistulas after stenting. Kozarek *et al*[13] did not find an association between perforation, bleeding or TE fistula development in patients who had received previous chemotherapy/irradiation. Consistent with the findings reported by Kozarek *et al*[13], TE fistulas did not develop in any of our patients who were receiving treatment.

Stent migration may be a bothersome problem in SEMS. Studies have reported the incidence of late migration from 0% to 58% for different types of covered stents. A higher incidence of late migration of all types of covered stents was observed compared to the uncovered types[6,18,21,40]. Stent migration was noted in 4 (4.4%) of our patients; 3 of these patients had covered and one had an uncovered type of stent.

Another ongoing issue with stents is the occurrence of recurrent dysphagia because of stent migration, tumoral or nontumoral tissue growth and food impaction. Frequencies of recurrent dysphagia associated with overgrowth, ingrowth and obstruction due to food impaction

reported in the literature varies between 17% and 33%, somewhat higher than those observed in our study[2,7]. As the malignant tumor continues to grow after stent implantation, the growth of tumor tissue through the stent lumen (ingrowth) and extension over the stent borders (overgrowth) are 2 major late complications[10,41,42]. The disadvantages of uncovered stents are tumor ingrowth and recurrent obstruction. Covered stents have been designed to prevent ingrowth. Ingrowth in uncovered stents has been reported in literature to be in the range of 0%-100%[6,10,11,38,39]. Authors who used partially covered stents reported the incidence of growth in the uncovered ends of stents between 2% and 25% in different series[10,41,42]. The frequency in covered stents has been reported as 0%-53%[2,6]. In our study, tumor ingrowth was found in 3 patients (3.3%) treated with uncovered stents.

Overgrowth is the result of progression of malignancy rather than a failure or a complication of the stent. It is usually seen in 2.3%-30% of patients after a mean period of 2-4 mo after stent replacement[2,6,9,39,43]. Overgrowth may not always be due to the tumor but may also result from benign epithelial hyperplasia or granulation tissue. The most frequent symptom in cases of overgrowth was dysphagia in our group. Following stenting, 6 patients (6.6%) were restented due to restenosis associated with proximal overgrowth. Restenosis associated with early proximal overgrowth occurred in only one of our patients (at day 13). Stent migration may facilitate development of overgrowth formation though it may not be possible to confirm this complication endoscopically. The use of covered stents may help decrease tumor ingrowth, although it will not affect tumor overgrowth. The most frequently used method for the treatment of tumor overgrowth is the placement of a second stent. Argon plasma coagulation was performed additionally in one of our patients with proximal tumor overgrowth. Stent obstruction was observed in 4 of our patients as a result of food impaction, and stents were cleaned endoscopically in all patients.

Hematemesis is also a possible complication after SEMS insertion, and the incidence in our study was 1.1% (1/90). This complication could have been the result of pressure necrosis, the natural progress of the disease, or esophageal or gastric trauma from the sharp end of the stent.

Mean survival after stenting, reported in the literature, varies between 53 and 198 d[6,7,12]. In our study mean survival after stenting was 134 d. Overall survival time in our study was not significantly different from others in the literature. It has been shown that survival is influenced by the type and stage of the underlying disease.

It has been reported that SEMS are being implanted in the absence of fluoroscopic guidance in some centers and that this is a safe approach[5,6,15]. We believe that esophageal stenting under fluoroscopic guidance with endoscopic control is safer.

In summary, in our retrospective screening, we noted that all of our patients had been stented with covered or uncovered metallic stents and that the use of these stents was safe and effective in rapid alleviation of dysphagia. The placement of a self-expandable metallic stent can improve the oral alimentation status. Esophageal fistulas can be completely and rapidly closed with placement of covered metallic endoprostheses. Esophageal stents are a means to an end: in the setting of malignant disease, they occlude TE fistulae or alleviate dysphagia, but do not affect the natural course of the disease process except by virtue of inadequate palliation or subsequent stent-related complications.

COMMENTS

Background

Self expandable metallic stents (SEMS) introduction plays an important role in the management of esophageal obstruction and fistula secondary to malignancy. Early palliation of dysphagia is important for the maintaining of nutritional status and life quality. SEMS provides a considerable palliation with low mortality and morbidity especially in patients with advanced disease.

Research frontiers

This research is regarding to the efficacy and safety of SEMS in patients with esophageal obstruction or fistula due to advanced cancer.

Innovations and breakthroughs

The authors found that the covered and uncovered SEMS are equally effective for dysphagia palliation in malignant esophageal obstruction. This study is the most comprehensive retrospective review from Turkey about the palliation of malignant esophageal obstruction and fistula with SEMS. Also this study showed that it is possible to minimize the complications which may arise from SEMS placement.

Applications

The advantages of SEMS over the other palliative methods are rapidly improving of dysphagia, nutritional status and life quality. Stenting with covered SEMS is the most effective method in patients with malignant esophageal fistula.

Peer review

Palliation of malign obstruction of esophagus *via* SEMS is a rapid and effective treatment. The authors summarized their own experience retrospectively and the manuscript is generally well written.

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BRIEF ARTICLE

Antimicrobial susceptibility of *Helicobacter pylori* strains isolated from patients in Shiraz, Southern Iran

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Abstract

AIM: To improve our understanding of Iranian regional variation in *Helicobacter pylori* (*H. pylori*) antibiotic resistance rates to find the best antibiotic therapy for eradication of *H. pylori* infections.

METHODS: A total of 266 patients undergoing endoscopy in Shiraz, Southern Iran, were included in this study. *H. pylori* strains were isolated from antral biopsies by culture and confirmed by the rapid urease-test and gram staining. Antibiotic susceptibility of *H. pylori* isolates was determined by E-test.

RESULTS: A total of 121 *H. pylori* strains were isolated, 50 from male and 71 from female patients. Data showed that 44% (n = 53), 20% (n = 24), 5% (n = 6), and 3% (n = 4) of all strains were resistant to the antibiotics metronidazole, amoxicillin, clarithromycin, and tetracycline, respectively. When the antibiotics were considered together we found 11 sensitivity patterns for the strains. Resistance to metronidazole was significantly higher in female than in male patients ($P < 0.05$). In about 71% of the metronidazole-resistant isolates, the minimum inhibitory concentrations (MICs) exceeded 256 μg/mL.

CONCLUSION: We found a moderate rate of primary resistance to metronidazole. However, a high MIC (> 256 mg/L) which was found in 71% of the isolates is considerable. In the case of amoxicillin, an increased resistance rate of 20% is worrying. Resistance to clarithromycin and tetracycline is also emerging among the *H. pylori* strains in our region.

Key words: Gastric disorders; *Helicobacter pylori*; Iran; Sensitivity; Treatment

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is involved in the pathogenesis

of a number of gastrointestinal diseases, including acute and chronic gastritis, peptic ulceration, gastric carcinoma and gastric lymphoma[1]. Eradication treatment of *H. pylori* infection usually consists of various combinations of drugs. Most commonly, an acid suppressor (usually a proton pump inhibitor) or an H_2-receptor antagonist (e.g. ranitidine) is prescribed in combination with two antibiotics usually amoxicillin, metronidazole or clarithromycin[1]. The combination of two antibiotics can increase the success of eradication therapy and decrease the possibility of secondary antibiotic resistance[2]. Antibiotic resistance in *H. pylori* is the major cause of eradication failure. Growing resistance often parallels the patterns of antibiotic consumption, and may vary within patient groups according to the geographic region, patient age and sex, type of disease, birthplace, other infections and other factors. The geographic map and the process of primary *H. pylori* resistance are clinically important, and should be considered when choosing eradication regimens, as is constant monitoring at both national and global level in an attempt to reach the recently recommended goal of eradicating more than 95% of resistant cases[3]. The prevalence of clarithromycin, metronidazole and amoxicillin resistance varies between countries and is highest for metronidazole[4,5]. Resistance to tetracycline and ciprofloxacin has been reported but appears uncommon[6-8].

There are several problems with antimicrobial susceptibility testing of *H. pylori*[9,10]. Agar or broth dilution methods are difficult to perform routinely[11], thus, disk-diffusion testing is often used because it is simple, easy to perform, and economical[12]. However, the E-test has proved to be an accurate method for assaying the susceptibility of fastidious organisms, including *H. pylori*, to antibiotics. The E-test has a more stable pattern of antibiotic release and has been found to tolerate prolonged incubation[13]. This is the main reason why the E-test rather than the disk diffusion method, has been recommended for *H. pylori*.

Pre-treatment resistance rates in *H. pylori* vary markedly between countries and regions. In Europe, mean resistance rates of 27% for metronidazole and 10% for clarithromycin are typical[14,15]. There is no systematic surveillance of primary antibiotic resistance rates in Shiraz, and widely divergent rates have been reported in Iran, depending on the local population.

The present study aimed to improve our understanding of the Iranian regional variation in *H. pylori* antibiotic resistance rates in relation to gender, and to find the best antibiotic therapy for the eradication of *H. pylori* infections.

MATERIALS AND METHODS

The patient groups and sample collection

In this study, 266 patients attending the endoscopy ward of Motahhary Clinic of Shiraz University of Medical Sciences during the period between October 2008 and October 2009 were enrolled. Exclusion criteria for patient recruitment to the study were: previous attempts to eradicate *H. pylori*, use of antibiotics or proton pump inhibitors within the last 2 wk prior to endoscopy, and previous gastric surgery. The diagnosis of *H. pylori* infection and confirmation of gastric disease by histology were established by a central study pathologist. Antral biopsies taken from each patient were transferred to the lab in an appropriate transfer medium (brain heart infusion broth, supplemented with 20% glucose) for *H. pylori* isolation and identification.

Isolation and identification of H. pylori

Biopsy samples were gently homogenized and cultured on rapid urease-test media and colombia agar base (Merck, Germany), supplemented with 10% lysed horse blood and 7% fetal calf serum and the antibiotics amphotericin B (5 μg/L), trimethoprim (5 μg/L) and vancomycin (10 μg/L). The cultures were kept in a microaerophilic atmosphere (7% O_2, 7.1% CO_2, 7.1% H_2, 79.8% N_2), provided by Anoxomate (Mark Ⅱ, Mart Microbiology BV, Netherlands) at 37℃ for 2-4 d. The isolates were then confirmed as *H. pylori* by positive oxides, catalase and rapid urease-tests. The samples were also evaluated for the presence of *H. pylori* by the modified gram staining and rapid urease-tests. If any of the two tests were positive simultaneously, the sample was considered *H. pylori* positive.

Antibiotic susceptibility test

For *in vitro* susceptibility testing of the *H. pylori* strains, a suspension equal to the McFarland tube no. 3 was prepared for each isolate. We used only one colony from each patient for the analysis. Brain heart infusion broth (Merck, Germany) plates, supplemented with fetal calf serum (Gibco, USA) were inoculated by confluent swabbing of the surface with the adjusted inoculum suspensions. The E-test strips (Biomerieux, France) for the antibiotics amoxicillin, metronidazole, tetracycline, and clarithromycin, were aseptically placed onto the dried surface of inoculated agar plates. The plates were then incubated at 37℃ under microaerophilic conditions. The minimum inhibitory concentrations (MICs) were read after 48-72 h of incubation on the basis of the intersection of the elliptical zone of growth inhibition using the MIC scale on the E-test strip, as per the manufacturer's instructions[16]. Susceptibility results were recorded as resistant according to the following interpretive criteria; for metronidazole, clarithromycin, tetracycline and amoxicillin, MIC breakpoints of $\geqslant$ 8 mg/L, $\geqslant$ 1 mg/L, $\geqslant$ 4 mg/L and $\geqslant$ 0.5 mg/L, respectively[17-19].

Statistical analysis

Fisher's exact test and *P* values were determined. A *P* value of < 0.05 was considered significant.

RESULTS

A total of 121 *H. pylori* strains were isolated from the patients under study, 50 from males and 71 from females. The antimicrobial susceptibility results of the *H. pylori* strains are presented in Table 1. According to the data, 44% (n = 53), 20% (n = 24), 5% (n = 6), and 3% (n = 4) of the strains were resistant to metronidazole, amoxicillin, clarithromycin, and tetracycline, respectively. Fifty isolates were

Table 1 Rates of antibiotic resistance in *Helicobacter pylori* isolates in relation to patient gender

Sex No.	No. of isolates (% of resistance)			
	MTZ	AMX	CLA	TET
Male (n = 50)	17 (34)	9 (18)	1 (2)	1 (2)
Female (n = 71)	36 (50)	15 (21)	5 (7)	3 (4)
Total (n = 121)	53 (43)	24 (19)	6 (5)	4 (3)

MTZ: Metronidazole; AMX: Amoxicillin; CLA: Clarithromycin; TET: Tetracycline.

Table 2 Antibiotic resistance patterns of the *Helicobacter pylori* strains

Antibiotic resistance patterns	*n*	Male	Female
MTZ	44	15	29
AMX	14	7	7
CLA	1	0	1
TET	1	1	0
MTZ-AMX	3	1	2
MTZ-TET	1	0	1
AMX-TET	1	0	1
AMX-CLA	1	0	1
MTZ-AMX-TET	1	0	1
MTZ-AMX-CLA	4	1	3
Sensitive	50	25	25
Total	121	50	71

MTZ: Metronidazole; AMX: Amoxicillin; CLA: Clarithromycin; TET: Tetracycline.

sensitive to all the tested antibiotics. When the antibiotics were considered together, we found 11 sensitivity patterns for drug sensitivity among the strains (Table 2). When the data were analyzed on the basis of patient gender (Table 1), 32% (n = 16), and 51% (n = 36) of the strains isolated from males and females, respectively, were resistant to metronidazole. Statistical analysis showed that resistance to metronidazole was significantly higher in female than in male patients ($P < 0.05$). With regard to clarithromycin and amoxicillin, the percentage of resistance in female patients was 7% and 20%, respectively and in male patients was 2% and 20%, respectively. The differences in resistance to these two antibiotics among the strains isolated from both genders were not significant ($P > 0.05$). We found that 3 (4%) and 1 (2%) strains isolated from female and male patients were resistant to tetracycline, respectively.

In about 71% of the metronidazole-resistant isolates, the MICs exceeded 256 μg/mL. The MIC ranges for the antibiotics tested in both genders are shown in Table 3.

Table 3 Range of minimum inhibitory concentrations for antibiotics tested against *Helicobacter pylori* strains in relation to patient gender

Sex	Range of MICs for antibiotics (mg/L)			
	MTZ	AMX	CLA	TET
Male	0.064 to > 256	< 0.016 to > 256	< 0.016 to 64	0.016 to 8
Female	0.047 to > 256	< 0.016 to > 256	< 0.016 to > 256	0.016 to 8

MIC: Minimum inhibitory concentration; MTZ: Metronidazole; AMX: Amoxicillin; CLA: Clarithromycin; TET: Tetracycline.

DISCUSSION

Resistance to antimicrobials is of particular concern to practitioners in this field, and is a major cause of the failure to eradicate *H. pylori* infections[20,21]. It has also been shown that resistance to different antibiotics develops in *H. pylori* strains by acquiring chromosomal mutations at the site where the drug acts[22]. However, many reports have indicated that the prevalence of resistance varies geographically and that there is a broad range of resistance variability depending on the drug used[23]. The special nutritional and atmospheric conditions required by these organisms make susceptibility testing relatively difficult; however, the E-test technique developed to determine the minimum inhibitory concentration (MIC) has remained valid[24]. Accordingly, in the present study we evaluated the sensitivity of *H. pylori* strains isolated from patients with gastric disorders to 4 antibiotics using the E-test to find the resistance pattern in these strains in our region.

It was observed that 44% of the isolates in this study were resistant to metronidazole with a MIC range of 0.064 to > 256 μg/mL. This resistance rate was consistent with reports from some developed countries, where it has been reported that 15.8%-40% of *H. pylori* strains were resistant to metronidazole[25-27]. However, most reports from developing countries describe a high level of resistance to metronidazole, which varies from 66.2% to 100%[28,29]. Resistance rates to metronidazole may also vary within a country. For example, in India, the resistance rate to metronidazole was high in Lucknow, Chennai and Hyderabad (68%, 88.2% and 100%, respectively), whereas a moderate rate was observed in Delhi (37.5%) and Chandigarh (38.2%)[30]. Similarly, the resistance rate was high (78%) in Tehran, Iran in one study carried out by Falsafi *et al*, while in another study it was reported to be 34%[31,32]. In Europe, according to studies conducted between 1989-2001 and 1990-2002, respectively, the resistance rate varied between 16.0% and 43% in pediatric patients and between 14.9% and 40.3% in adult patients[22]. It seems that primary resistance to nitro-imidazole has been attributed to frequent use of the drug, which is commonly prescribed for other diseases, especially parasitic conditions, and periodontal or gynecological infections. The higher resistance rate to metronidazole in females reported in this and other studies could be due to the treatment of gynecological infections using this drug which is also used in the treatment of bacterial vaginosis. Moreover, the use or abuse of this inexpensive drug may contribute to the increased metronidazole resistance seen in developing countries[33]. For this reason, metronidazole has been excluded from first-line empirical therapy plans in some countries[23]. However, it has been reported that the results of *in vitro* resistance to this drug are also poorly correlated with the outcome of therapy, and consequently, susceptibility testing is not rou-

tinely indicated[34]. Therefore, assessment of the drug concentration in blood samples could be recommended to assess the correlation with *in vitro* results. Another important finding from the present study was that in about 71% of the metronidazole-resistant isolates, the MICs exceeded 256 μg/mL, which has rarely been reported[17].

In contrast to most studies, we found a high resistance (20%) to amoxicillin among *H. pylori* isolates. Most studies have shown that *H. pylori* resistance to this drug is either very rare or non-existent[25,33,35]. Usually, the MIC of amoxicillin for *H. pylori* is very low (0.03 μg/mL); nevertheless, in our study, we found a few sensitive isolates with reduced susceptibility (MICs: < 0.016 μg/mL)[33]. However, high resistance rates have been reported in some studies from other parts of the world: 18.5% in South Korea[36], 19.4% in Indonesia[37], 32.8% in India[38], and 38% in Brazilia[39]. In a study conducted in Ile-Ife, southwest of Nigeria, 100% of the 32 isolates were resistant[28]. It has been shown that resistance to amoxicillin could have emerged by genomic mutation in the pbp1A gene[39]. When comparing different sets of data, it is important to note that variations in rates may arise due to the effects of inter-laboratory reproducibility, caused by the lack of standardized testing protocols or regional prescribing practice. This may be the reason why the resistance rates for amoxicillin in *H. pylori* isolates have been reported to be 1.6% and 27% in different studies conducted in Iran[31,32]. Moreover, high resistance to amoxicillin observed in the present study reflects the importance of its use in our country.

Clarithromycin is a macrolide used frequently in combination with other antimicrobial agents for the treatment of *H pylori* infection[40]. However, resistance to clarithromycin has become one of the major reasons for treatment failure[41]. The prevalence of *H. pylori* resistance to clarithromycin varies in different countries, and was 12% in Japan, 1.7%-23.4% in Europe and 10.6%-25% in North America[22]. Resistance in 5% of our isolates resembles data from the Northern regions of Europe[27]. In contrast, two other studies from Tehran, Iran reported a high rate of resistance to clarithromycin ranging from 16.7% to 21%[31,32]. Since clarithromycin is not currently used in Iran, emerging resistance to this antibiotic is unexpected. On the other hand, it has been shown that there is cross-reactivity between clarithromycin and other macrolides such as erythromycin, which implies that resistance to one macrolide could cause the emergence of resistance to other macrolides[42]. Genetic studies have revealed that clarithromycin resistance is often associated with point mutation of the 23S rRNA[2].

We observed a low resistance (3%) to tetracycline among the isolates, which is consistent with most studies which have reported no resistance or low resistance to this antibiotic in *H. pylori* strains[2,31,35,43]. In contrast, a high resistance rate (20%) to tetracycline was reported by Falsafi *et al*[32]. Similarly, these variations could be due to the effects of inter-laboratory reproducibility caused by the lack of standardized testing protocols or regional prescribing practice. However, resistance to tetracycline, mainly caused by mutations in the 16S rRNA gene, is emerging and can impair the efficacy of such second-line regimens[44]. Thus, it seems that molecular methods can help verify the exact rate of resistance to this antibiotic. Recently a novel real-time PCR has been described which is able to detect the strains carrying the mutant genes for tetracycline resistance[44].

In conclusion, this study showed a moderate rate of primary resistance to metronidazole which is included in the guidelines for the empirical therapy of *H. pylori* infections. However, a high MIC (> 256 mg/L) observed in about 71% of the isolates is considerable. On the other hand, in the case of amoxicillin, there was an increased resistance which is worrying. In particular, it is important to determine whether the increased resistance to amoxicillin is a result of its increased use or due to the ethnic differences of the populations described herein. The data also indicate that resistance to clarithromycin and tetracycline is emerging among the *H. pylori* strains in our region. Therefore, considering the increasing resistance rate in many countries, monitoring of susceptibility of *H. pylori* to these antibiotics appears to be necessary in order to choose effective therapy to eradicate *H. pylori* infections and to optimize the regimen in case of treatment failure. Finally, taking into account the present findings along with other reported findings, continued surveillance of the resistance profiles and the resistance mechanisms present in *H. pylori* strains isolated in Iran is essential, if therapeutic plans are to satisfy the country's needs.

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COMMENTS

Background

Antibiotic resistance in *Helicobacter pylori* (*H. pylori*) is the major cause of eradication failure. Growing resistance often parallels the patterns of antibiotic consumption, and may vary within patient groups according to the geographic region, patient age and sex, type of disease, birthplace, other infections and other factors.

Research frontiers

Geographic mapping and the process of primary *H. pylori* resistance are clinically important, and should be considered when choosing eradication regimens. These should also be constantly monitored both at national and global level in an attempt to reach the recently recommended goal of eradicating the highest rate of resistance. The present study aimed to improve our understanding of Iranian regional variation in *H. pylori* antibiotic resistance rates in relation to gender and to find the best antibiotic therapy for the eradication of *H. pylori* infections.

Innovations and breakthroughs

The E-test has proven to be an accurate method of assaying the susceptibility of fastidious organisms, including *H. pylori*, to antibiotics. Using this method and improved culture conditions, we found a moderate rate of primary resistance to metronidazole which is included in the guidelines for the empirical therapy of *H. pylori* infections. However, a high minimum inhibitory concentration (MIC) (> 256 mg/L) observed in 71% of the isolates is considerable. On the other hand, in the case of amoxicillin, there was an increased resistance (20%) which is worrying. The data also indicate that resistance to clarithromycin and tetracycline is emerging among the *H. pylori* strains in our region.

Applications

Considering the increasing rate of resistance in many countries and based on the varied results from different studies, even in the same regions, the results of this study can improve the monitoring of *H. pylori* susceptibility to antibiotics, which is necessary in order to choose effective therapy to eradicate *H. pylori* infections and to optimize the regimen in case of treatment failure in our region.

Terminology

MIC is minimum inhibitory concentration. E test is the epsilometry test, a test to determine the MIC of antimicrobial agents using strips with epsilometric concentrations of antimicrobials.

Peer review

It is an interesting publication showing the prevalence of antibiotic resistance of *H. pylori* in southern part of Iran (Shiraz).

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BRIEF ARTICLE

Expression and purification of a functional human hepatitis B virus polymerase

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Abstract

AIM: To identify a method for efficient large-scale purification of functional hepatitis B virus polymerase (HBV-Pol) without addition of cellular factors.

METHODS: Full-length HBV-Pol (843 amino acids) tagged with 5′ end Polyhistidine was expressed at a high level in an *Escherichia coli* (*E. coli*) system. Sodium dodecyl sulfate lysis buffer was utilized to dissolve insoluble HBV-Pol, and Ni-NTA resin affinity chromatography was utilized for HBV-Pol purification. Most recombinant HBV-Pol was eluted with 100 mmol/L imidazole in the presence of NP-40, a weak detergent that keeps HBV-Pol in solution. A reducing agent was utilized throughout the purification steps to keep soluble HBV-Pol from redundant disulfide bond formation.

RESULTS: The large-scale production of functional intact human HBV-Pol was achieved in an *E. coli* expression system. Purified HBV-Pol showed stable reverse transcriptase activity and DNA polymerase activity. The purified protein was of high purity and had stable reverse transcriptase activity.

CONCLUSION: Large-scale production of HBV-Pol in pure form should facilitate crystallization and detailed analysis of the structure and mechanism of HBV-Pol. Ability of this purification approach to obtain human HBV-Pol in an enzymatically active form should be helpful for development of drugs for treatment of chronic hepatitis B.

Key words: Hepatitis B Virus; Virus polymerase; Reverse transcriptase; Detergent

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health problem. It is estimated that between 350 and 400 million people worldwide are chronically infected, and a significant proportion of chronic infection patients ultimately develop life-threatening liver disease such as cirrhosis, hepatocellular carcinoma (HCC) and other complications[1]. HBV replicates *via* a reverse transcription step, using the polymerase (HBV-Pol) that is encoded by its own genome. HBV-Pol is a multifunctional protein, with protein-priming activity[2-4],

DNA polymerase, reverse transcriptase[2,5] and RNase H activity, but it is short of proofreading activity[6]. Most approved medications for chronic hepatitis B (CHB) infection are nucleotide reverse transcriptase inhibitors (NRTIs) that target HBV-Pol[7]. Although NRTIs have been used in CHB infection for several decades, their therapeutic efficacy has been limited by high frequency appearance of mutants during treatment[8]. Therefore, a quick and easy way to obtain a large quantity of functionally intact human HBV-Pol is required for selection of sensitive CHB medications, mutated HBV strains research, and mass high-throughput screening.

It is known that expression of an enzymatically active HBV-Pol in heterologous systems, or purification of useful quantities of human HBV-Pol from virions is difficult to achieve. Due to these problems, drug development for HBV infection has not progressed satisfactorily, and biological studies of hepadnaviral polymerase have been conducted by using duck HBV[9]. Several groups have succeeded in achieving heterologous *in vitro* expression of full-length polymerase proteins of duck HBV that exhibit DNA-dependent DNA polymerase (DDDP) activity and RNA-dependent DNA polymerase (RDDP) activity. However human HBV-Pol is expressed by *in vitro* translation with a rabbit reticulocyte lysate system[10], and *in-vitro*-translated human HBV-Pol shows only DDDP activity and fails to show RDDP activity. RDDP activity of human HBV-Pol has been observed in *Escherichia coli* (*E. coli*) as a fusion protein in frame with maltose-binding protein[11]. The enzymatically active HBV-Pol has also been obtained in *E. coli* by co-expression of the polymerase with the chaperone GRP94[12]. However, the stable and large-scale heterologous expression of intact human HBV-Pol without co-expression of molecular chaperon in common hosts such as *E. coli* or yeast has not been reported.

In this study, a full-length HBV-Pol with a 6 × His tag was expressed in *E. coli*. HBV-Pol is a large molecule with approximately 2.5% cysteine residues[2], therefore, the protein is expected to be present as inclusion bodies. For this reason, the total lysate was dissolved by applying high concentration of sodium dodecyl sulfate (SDS) and reducing agents to dissolve inclusion bodies, and then SDS was replaced by weak detergent for renaturation during washing. Finally, the target protein was purified with nickel-based chromatography. Purified HBV-Pol showed RDDP and DDDP activity. This is believed to be the first time that functional intact human HBV-Pol has been expressed in *E. coli* without co-expression molecules or in the presence of certain helper chaperons. The functional HBV-Pol might be helpful for development of potential pharmaceutical agents for CHB treatment.

MATERIALS AND METHODS

Plasmid construction

Liver tissues were obtained from a chronic hepatitis B surface antigen carrier who developed HCC and underwent surgical resection. The tumor tissues were dissected and immediately cut into small pieces and stored into liquid nitrogen until use. The cellular DNA was isolated from tissues by SDS-protease K digestion and phenol-chloroform extraction as described previously[13]. HBV-Pol sequence [spanning 2307 to 1623 bp, 843 amino acids] were amplified with PrimeSTAR™ high fidelity polymerase using CPNotIF01: GTTGCGGCCGCATA-ATGGCCCTATCTTATC and CPBstBIR01: ATTTTC-GAATTCTCACGGTGGTTTCCA for complete P gene. The vectors were designed as follows (Figure 1).

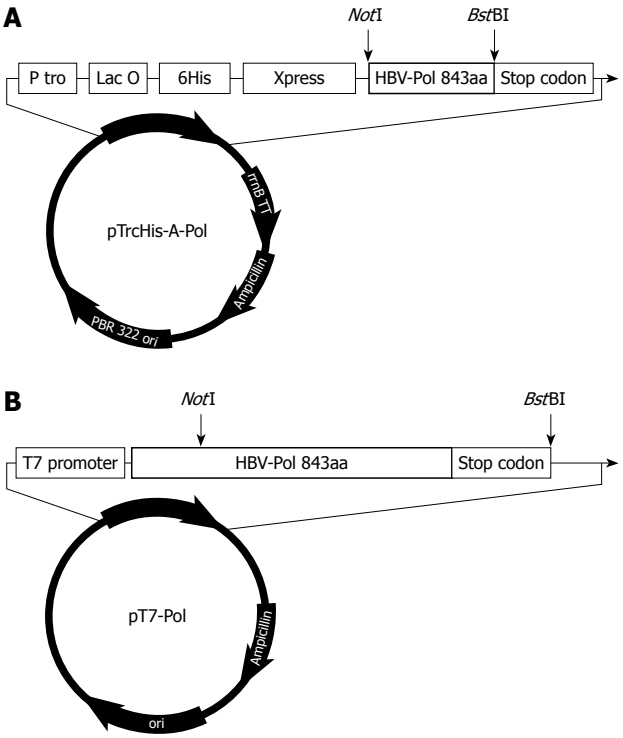

Figure 1 Organization of pTrcHis-A-Pol and pT7-Pol recombinant plasmids. A: Structural arrangement of pTrcHis-A-Pol. A full-length hepatitis B virus polymerase (HBV-Pol) sequence was fused into pTrcHis-A between *Not*I and *Bst*BI sites under the control of Trc promoter and Lac operator. This polyhistidine tag plays a role in rapid purification using a nickel-based resin. To determine the expression level under different conditions, an Xpress antigen was fused to the 5'-end of the HBV-Pol sequence; B: Structure of pT7-Pol. A full-length HBV-Pol sequence was fused to P-T7 between *Not*I and *Bst*BI sites. Full-length HBV-Pol sequence was under the control of the T7 promoter for expression in the TNT T7 transcription–translation-coupled rabbit reticulocyte lysate expression system.

The pTrcHis-A-Pol and pT7-Pol were constructed by the following procedure. HBV-Pol full-length sequence was cloned into *Not*I and *Bst*BI sites of the two expression vector pTrcHis-A (Invitrogen, Carlsbad, CA, USA) and pT7 vector (Promega, Madison, WI, USA). For expression of HBV-Pol in *E. coli*, the plasmid pTrcHis-A-Pol was constructed as showed in Figure 1A. The HBV-Pol frame was under the control of the Trc promoter and Lac operator. His-tag and Xpress antigen were fused at the 5' end of the HBV-Pol sequence. For expression of HBV-Pol in the rabbit reticulocyte lysate system, the plasmid pT7-Pol was constructed as showed in Figure 1B. The HBV-Pol frame was driven by T7-promoter.

E. coli transformation

Plasmid pTrcHis-A-Pol was chemically transformed in competent DH5α (*F-,φ80dlacZ⊿M15,⊿(lacZYA-*

*argF)U169,deo*R,*recA1,endA1,hsdR17(rk-,mk+),phoA, supE44,λ-,thi-1,gyrA96,relA1*) cells following the manual supplied by Real Biotech Corporation (Taipei, Taiwan, China). Isopropylthiogalactopyranoside (IPTG, Sigma, St Louis, MO, USA) was added into 5 mL overnight-cultured *E. coli*, to a final concentration of 1 mmol/L, and incubated for 4, 8, 12 and 16 h, respectively, at 18℃, 24℃, 30℃, 37℃ and 42℃, as indicated. Cells were harvested by centrifugation and disrupted with lysis-buffer (50 mmol/L phosphate buffer, pH 8.0; 0.5 mmol/L NaCl; 1% SDS; 10 mmol/L imidazole; 0.1 mg/mL lysozyme; 20 mmol/L 2-mercaptoethanol; 1 × Roche protein inhibitor cocktail). Protein expression level was determined with dot blot using anti-Xpress antibody (Invitrogen).

Expression and purification of histidine-tagged HBV-Pol

Transformed cells were grown in 100 mL LB broth until the culture reached OD_{600} 0.6-0.8. IPTG was added to a final concentration of 1 mmol/L and incubated for 8-10 h at 18℃ with shaking. Induced cells were harvested by centrifugation at 2000 *g* for 20 min at 4℃. The ratio of lysis-buffer volume and wet weight of cell pellet was about 4 to 1. The lysate was incubated at room temperature for 30 min and sonicated for 10 × 10 s. The samples were cooled on ice for 5-10 s between each sonication. The suspension was centrifuged at 20000 *g* for 30 min at room temperature. The supernatant was transferred to an Ni-NTA resin (Invitrogen) column (bed volume: 2 mL), which had been equilibrated with 16 mL equilibration buffer (same components of previous lysis buffer without lysozyme). Resin and lysate supernatant were mixed thoroughly but gently for 45-60 min at room temperature. The resin was washed with 8 ml washing buffer (50 mmol/L phosphate buffer, pH 8.0, 0.5 mmol/L NaCl, 1% NP-40, 10 mmol/L imidazole, 20 mmol/L 2-mercaptoethanol, and 1 × Roche protein inhibitor cocktail) for 6-8 times. The HBV-Pol fractions were eluted with 6 mL E-50, E-75, and E-100 Elution buffer (50 mmol/L phosphate buffer, pH 8.0, 0.5 mmol/L NaCl, 1% NP-40, 1 × Roche protein inhibitor cocktail), and dithiothreitol (DTT) was added to the harvest tube to achieve a final concentration of 5 mmol/L. The concentration of imidazole in E-50, E-75, and E-100 buffer was 50 mmol/L, 75 mmol/L and100 mmol/L, respectively. HBV-Pol elution fractions were harvested and stored at -70℃. The concentration of purified protein samples was determined using a BCA assay with bovine serum albumin as a standard.

Anti-reverse transcriptase polyclonal antibody preparation

Anti-reverse transcriptase (RT) polyclonal antibody was prepared before full-length polymerase purification (data not shown). A recombinant RT domain (spanning 304-693 amino acids) was purified from *E. coli*. Fifty micrograms purified RT and Freund's complete adjuvant were injected subcutaneously into C57BL/6 mice (Macrogen, Seoul, South Korea). Another 50 μg purified RT and Freund's incomplete adjuvant were injected 2 wk after the first injection. The mice were sacrificed 2 wk after the second injection for serum extraction of anti-RT polyclonal antibody. The antibody titers against the RT peptides, monitored with ELISA (Sigma), were > 1:32000.

SDS-PAGE, dot blot and Western blotting analysis

Total lysate and other washing/elution fractions were heated at 95℃ for 5 min for denaturation. Proteins were separated by SDS-PAGE. Gels were stained with Coomassie Blue. For dot blot analysis, 1 μL of each sample was dropped on nitrocellulose membranes (Amersham, Bucks, UK), and the membranes were treated first with a 1/4000 dilution of anti-Xpress antibody and then goat-anti-mouse conjugated alkaline phosphatase (AP; Invitrogen). For Western blotting analysis, proteins were electrophoretically transferred to a polyvinylidene difluoride blotting membrane (Amersham, Piscataway, NY, USA), and membranes were treated first with a 1/4000 dilution of anti-RT antibody and then goat-anti-mouse conjugated AP.

Expression of HBV-Pol in in vitro transcription and translation system

The recombinant plasmid pT7-Pol was purified with Qiagen Midiprep DNA purification kit. *In vitro* transcription and translation reactions were performed using the TNT T7 coupled reticulocyte lysate system (Promega). Two micrograms of the plasmid DNA template was transcribed and the protein was translated in each 50-mL reaction in the presence or absence of 40 mCi of [^{35}-S]-methionine (1000 Ci/mmol) (Amersham) at 30℃ for 75 min[10]. The *in vitro* translation reaction was stopped by the addition of 0.1 mg/mL cycloheximide for the polymerase activity assay, or SDS sample buffer for checking the efficiency of translation. The *in vitro* translated proteins were separated by 4%-12% SDS-PAGE and dried prior to autoradiography.

DNA polymerase activity and RT activity assays

DDDP and RT/RDDP were monitored by synthesis of DNA using poly (dA) • oligo $(dT)_{12-18}$ and poly (rA) • oligo $(dT)_{12-18}$ as template primer (Amersham Biosciences), respectively. The standard enzyme reaction (50 mL) contained 50 mmol/L Tris-HCl pH 7.4, 50 mmol/L KCl, 10 mmol/L $MgCl_2$, 1 mmol/L DTT, 0.01% Nonidet P-40, 50 ng homopolymer template [poly(dA) • oligo $(dT)_{12-18}$ for DDDP activity assay and poly(rA) • oligo $(dT)_{12-18}$ for RDDP activity assay], and 2 mCi of [α-^{32}P]dTTP (3000 Ci/mmol), (PerkinElmer, USA). For RDDP activity assay, RNase inhibitor and RNase-free water were employed in the reaction[10,11]. Reactions were started by the addition of 0.5 mg of the purified HBV-Pol or 5 μL products from the TNT-T7 coupled reticulocyte lysate system into the reaction buffer. The endogenous DNA polymerase activity from the reticulocyte lysate was suppressed by the addition of 60 mmol/L aphidicolin and 1 mmol/L NEM. After incubation at 37℃ for 75 min, reactions were stopped by the addition of 0.2 mg/mL protease K in the presence of 0.5% SDS. Incubation was continued for another 20 min, followed by spotting on Whatman DE81 filter paper. Filters were washed three times with 0.5 mol/L Na_2HPO_4, and once with distilled

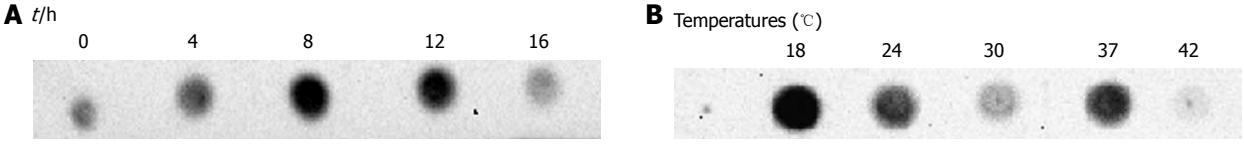

Figure 2 Induction optimization of hepatitis B virus polymerase expressed in *Escherichia coli*. Total lysate samples of *Escherichia coli* transformants after different induction times were blotted on to nitrocellulose membranes. Hepatitis B virus polymerase (HBV-Pol) expression level was determined by anti-Xpress antibody. A: 8-h induction sample showed the strongest signal; B: HBV-Pol expression level at different temperatures was determined by anti-Xpress. Sample from 18℃ showed the highest expression level. Preliminary expression data showed that no obvious signal was detected in samples induced at temperatures < 18℃, therefore, previous values for duration and temperature for induction were selected for this study.

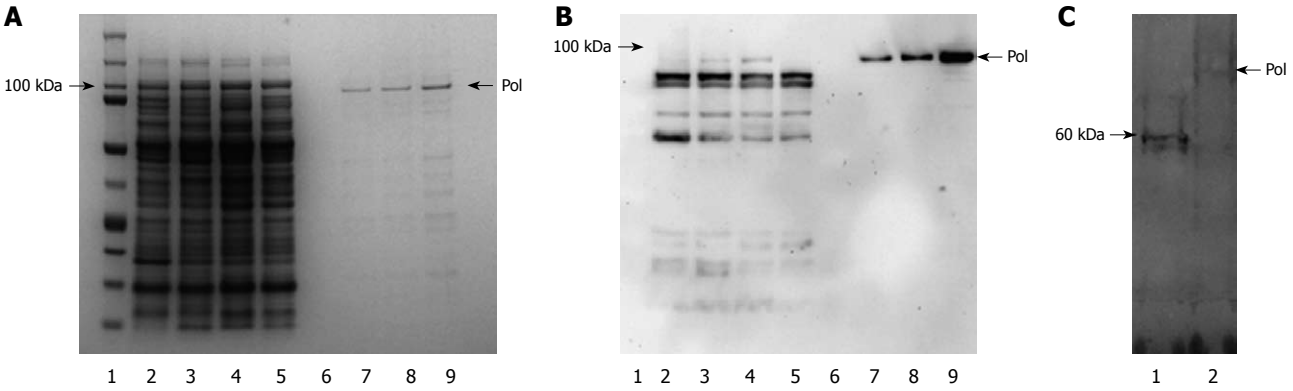

Figure 3 Expression and purification of hepatitis B virus polymerase. Recombinant human hepatitis B virus polymerase (HBV-Pol) was produced in *Escherichia coli* (*E. coli*) cells transformed by pTrcHis-A-Pol and purified with nickel-based resin. Protein samples were analyzed by 4%-12% sodium dodecyl sulfate (SDS)-PAGE, and gels were stained with Coomassie Blue (A). HBV-Pol bands were located at the expected molecular mass, approximate 98 kDa (lane 1). Although HBV-Pol bands were also detected in 50 mmol/L (lane 7) and 75 mmol/L (lane 8) imidazole washing fractions, most HBV-Pol was eluted by 100 mmol/L imidazole (lane 9). Purified recombinant human HBV-Pol was also analyzed by immunoblotting with an anti-reverse transcriptase (RT) antibody (B). HBV-Pol bands were detected in total lysate of uninduced transformant (lane 3) and total lysate of induced transformant (lane 4), but not in untransformed *E. coli* (lane 2), unbound fraction (lane 5) and fifth washing fraction with 10 mmol/L imidazole (lane 6). Some positive small bands were also detected in lanes 2-5, which was mainly because some components from total lysate of E. coli were recognized by anti-RT polyclonal antibody. Expression of human HBV-Pol in TNT T7 transcription-translation-coupled rabbit reticulocyte lysate expression system is shown in C. PT7-pol and pT7-luciferase (1650 bp, ORF size approximate 61 kDa) were added to *in vitro* transcription-translation reactions for 75 min at 30℃ in the presence of [^{35}S]-methionine. Five microliters of each sample was incubated in SDS sample buffer at 95℃ for 3 min, and electrophoresed through 4%-12% SDS-PAGE. A band of pT7-luciferase (lane 1) and pT7-pol (lane 2) were detected by autoradiography.

H_2O[11]. Incorporation of radioactivity was determined by liquid scintillation counting in a Packard Tri-Carb Series 2300 liquid scintillation counter.

RESULTS

Expression, induction optimization and purification of HBV-Pol in E. coli

A high expression level of target protein is crucial to obtain an ideal elution result in affinity chromatography. To achieve the highest expression level, expression level was examined at various conditions. Recombinant HBV-Pol is a large molecule of 95 kDa full-length HBV-Pol plus a 5-kDa fused tag, and a low induction temperature and short or long induction time were considered for expression. It is known that heterologous protein was stabilized at low temperature[14] and formation of inclusion bodies was restrained[15]. Decreasing the temperature of induction medium should lower the rate of association of folding intermediates, which allows unfolded proteins and partially folded proteins to have more time to refold into native, soluble tertiary structures. Appropriate induction time is another important factor to obtain sufficient recombinant polymerase. The quantity of expressed protein varies with different induction times.

HBV-Pol level was monitored in the total lysate of *E. coli* transformed with HBV-Pol recombinant plasmid, pTrcHis-A-Pol (Figure 2). HBV-Pol expression level was the highest at 8 h induction compared to shorter or longer incubation (Figure 2A). In terms of temperature, the lowest temperature tested, 18℃, showed the highest level of expression compared to other samples expressed at higher temperatures (Figure 2B). Based on these data, incubation of HBV-Pol expression was carried out under these conditions throughout the experiment. For HBV-Pol purification, a denaturing-renaturing purification protocol was applied. According to previous lysis data under native conditions, inclusion bodies do not dissolve in the presence of weak detergent at 4℃, which decreased the quantity of target protein in the binding step. A strong detergent (SDS) was employed in the lysis step to dissolve overexpressed HBV-Pol in inclusion bodies, and to denature some protease present in the total lysate.

HBV-Pol fractions were eluted with elution buffers (50, 75, 100 mmol/mL imidazole). Figure 3A shows that a prominent HBV-Pol band was detected with SDS-PAGE, and analysis by Western blotting confirmed the purification results (Figure 3B). DTT, a stronger reducing agent

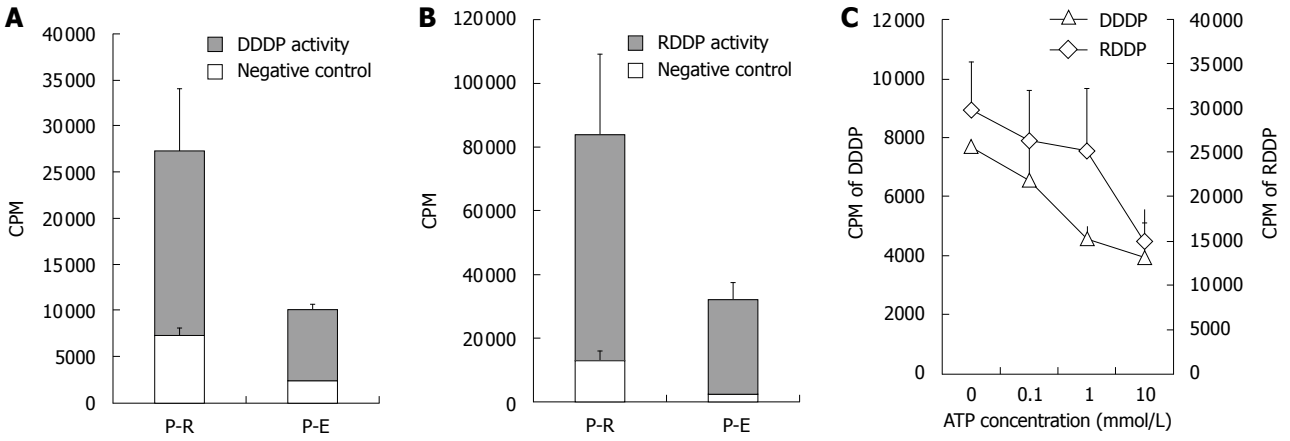

Figure 4 Enzyme activity assay of recombinant hepatitis B virus polymerase. Comparison of enzyme activities between hepatitis B virus polymerase (HBV-Pol) from *Escherichia coli* expression system (P-E) and *in vitro* expression system (P-R). DNA-dependent DNA polymerase (DDDP) activity and RNA-dependent DNA polymerase (RDDP) activity of the P-E and P-R were monitored under standard conditions. All endogenous DDDP activities were suppressed by 60 mmol/L aphidicolin and 1 mmol/L NEM (A and B). The effects of increasing concentrations of adenosine triphosphate (ATP) are shown in C. DDDP activity using poly (dA) • oligo $(dT)_{12-18}$ and RDDP activity using poly (rA) • oligo $(dT)_{12-18}$ were used as the substrate for DDDP activity and RDDP activity assays, respectively. CPM: Counts per minute.

than 2-mercaptoethanol, is suitable for long-term protein storage and is used in many experiments for HBV-Pol purification at a high concentration[2,5,6,16]. Although nickel resin is easily reduced by strong detergent, in the present study, 2-mercaptoethanol was employed in all steps except elution. DTT was finally added into HBV-Pol fractions for long-term storage. SDS-PAGE and Western blotting clearly showed high efficiency of purification. Involvement of the denaturing-renaturing purification process also suggested that purified recombinant HBV-Pol is a stable, single-component product. HBV-Pol was purified up to 5 mg/mL.

There have been many reports about purified or partially purified active HBV-Pol in eukaryotic systems. In the present study, recombinant HBV-Pol was also expressed in a reticulocyte lysate system as a positive control in the activity assay. The protein products from *in vitro* expression labeled with [^{35}S]-methionine are shown in Figure 3C. Approximately 95-kDa HBV-Pol protein was detected as predicted from the nucleotide sequence of the HBV-Pol-ORF.

Characterization of the purified HBV-Pol

To measure the enzymatic activity of purified HBV-Pol, the enzymatic activity assays for DDDP and RDDP were performed by using purified HBV-Pol (expressed in recombinant *E. coli*, named P-E) and HBV-Pol (expressed in reticulocyte system, named P-R). pT7 plasmid was expressed in the reticulocyte lysate system as a negative control for P-R enzyme activity assays. The solvent of P-E was regarded as a negative control for P-E enzyme activity assays. Enzyme activities of P-R and P-E were compared (Figure 4A-C). P-R showed medium DDDP activity and much higher RDDP activity under these reaction conditions. Homopolymer primer-template has been used for many groups[10,11] in HBV-Pol activity assays. Activity assay results showed that P-E and P-R have high RDDP activity and relatively weak DDDP activity compared to the negative controls. This pattern had been observed previously with partially purified HBV-Pol[9].

DDDP and RDDP activities affected with adenosine triphosphate

It has been shown that adenosine triphosphate (ATP) participates in HBV-Pol activation during duck HBV replication[17]. To study the effect of ATP on purified HBV-Pol, different concentrations of ATP were added to the enzyme activity assays. As shown in Figure 4B, RDDP and DDDP activities of P-R were affected by ATP concentration. In the presence of ATP (0.1, 1 and 10 mmol/L), RDDP and DDDP activities of P-R showed a declining trend in a dose-dependent manner.

DISCUSSION

Human HBV-Pol is crucial for HBV genome DNA replication. It shows RDDP activity in minus strand DNA synthesis and DDDP activity in plus strand synthesis. Among six medications approved by the United States Food and Drug Administration for the treatment of CHB, lamivudine, adefovir dipivoxil, entecavir and telbivudine are HBV-Pol inhibitors[7]. Blocking HBV DNA replication is still the main target for CHB therapy. However, stable and large-scale heterologous expression of intact human HBV-Pol in common hosts such as *E. coli* or yeast has not been successfully achieved[9]. Although recombinant HBV-Pol has been expressed in different systems and purified in various ways, obtaining high-purity functional HBV-Pol without coexpression of a chaperon is still problematic[2,5,9,12]. Therefore, we developed a reliable purification method for further investigation of HBV-Pol activities.

The major obstacle for a large multi-functional protein such as HBV-Pol is its instability in heterologous expression systems and during purification[9]. It is known that inclusion bodies are formed during expression of human HBV-Pol, not only in *E. coli* expression systems, but also in eukaryotic expression systems[2]. In accordance with this, the target protein was present in total lysate and pellet rather than supernatant in detergent-free lysis buffer. Our preliminary study showed that expressed human HBV-Pol was degraded easily

under native purification conditions, even with the presence of protease inhibitor. Choi *et al*[9] also have observed this phenomenon in a yeast expression system. Therefore, the key points of successful large-scale purification of human HBV-Pol are the solubilization and stabilization of the target protein. So far, nobody has reported a high level of expression and purification of HBV-Pol by using an *E. coli* expression system, in which we purified the protein up to 5 mg/mL. This is far higher than the earlier studies done by Choi *et al*[9], who used a *Pichia methanolica* expression system and Qadri *et al*[5] who used *Saccharomyces cerevisiae*.

In this study, we tried to achieve a high level of recombinant HBV-Pol with application of the following strategies. First, an amino-terminal 6 × His tag was used to facilitate rapid purification. Also, a sequence that encoded the Xpress epitope was fused to the N terminus of HBV-Pol so that it could be recognized rapidly by anti-Xpress primary antibody. Second, induction temperatures were lowered to maximize target protein expression. Third, for cell lysate purification, strong detergent lysis was followed by weak detergent washing and elution. Fourth, a high-concentration reducing agent was present during all the purification steps to keep HBV-Pol in soluble form by inhibiting disulfide bond formation, because approximately 2.5% of amino acid residues of HBV-Pol are cysteine.

It has been reported that structural and biochemical investigations of HBV-Pol have been complicated by the requirement of cellular factors such as HSP90[18], HSP60[3] and other cofactors[19] during purification. However, we developed a new method for large-scale purification of fully active HBV-Pol without addition of cellular factors by adjustment of salts during the purification steps. A binding step under denaturation conditions was used to isolate target protein from total lysate. With the presence of 1% SDS in the lysate, insoluble human HBV-Pol was dissolved rapidly. Proteases released from *E. coli* were also inactive in this SDS denaturing step. Soluble HBV-Pol was washed and eluted from nickel resin using the buffer in the presence of weak detergent and reducing agent. SDS was replaced by NP-40 during washing and elution. To prevent target protein from oxidization and to keep cysteine residues in a reduced state, a high concentration of reducing agents was employed throughout the purification steps.

The large-scale production of functionally intact human HBV-Pol was achieved in the *E. coli* expression system in this study. The availability of this recombinant protein in pure form should facilitate the crystallization and detailed analysis of the structure and mechanism of HBV-Pol. The availability of a large quantity of functional human HBV-Pol will help in high-throughput screening assays for development of potential pharmaceutical agents for CHB treatment.

COMMENTS

Background

Hepatitis B virus polymerase (HBV-Pol) is a multifunctional protein, which has intrinsic RNA-dependent reverse transcriptase (RT), DNA-dependent DNA polymerase, and RNase H activity. HBV-Pol is limited by difficulties in expressing and purifying the proteins in a heterologous system. This is believed to be the first time that functionally intact human HBV-Pol was expressed in *Escherichia coli* (*E. coli*) without co-expression of other molecules or in the presence of certain helper chaperons. Purified HBV-Pol showed stable RT activity and DNA polymerase activity. Functional HBV-Pol might be helpful for development of potential pharmaceutical agents for chronic hepatitis B (CHB) treatment.

Research frontiers

HBV is a small DNA virus that replicates by reverse transcription of pre-genomic RNA, and is a major threat to human health. Most approved medications for CHB infection are nucleotide reverse transcriptase inhibitors that target HBV-Pol. Analysis of HBV-Pol has been hampered by the inability to express the functional enzyme in a recombinant system. For this reason, we applied various strategies to the production of functionally intact human HBV-Pol on a large scale by using an *E. coli* expression system.

Innovations and breakthroughs

This is believed to be the first time that functionally intact human HBV-Pol has been expressed in *E. coli* without co-expression of other molecules or in the presence of certain helper chaperons. To achieve high levels of recombinant HBV-Pol, an N-terminal 6×His tag and Xpress epitope were fused to the N terminus of HBV-Pol. Second, the induction temperature was lowered to maximize target protein expression, and a high concentration of reducing agent was present during all the purification steps to keep HBV-Pol in a soluble form. The availability of this recombinant protein in pure form should facilitate the crystallization and detailed analysis of the structure and mechanism of HBV-Pol.

Applications

The availability of a large quantity of functional human HBV-Pol can be helpful in high-throughput screening assay for potential pharmaceutical agents for CHB treatment.

Peer review

This is a well-written paper and documents the expression and purification of full-length HBV-Pol in an *E. coli* system with promising results that could be the basis of forthcoming research on potential pharmaceutical agents for CHB treatment.

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BRIEF ARTICLE

Protective effects of *Lactobacillus plantarum* against epithelial barrier dysfunction of human colon cell line NCM460

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Abstract

AIM: To investigate the effects of *Lactobacillus plantarum* (*L. plantarum*) in the intestinal permeability and expression of tight junction (TJ) using the normal human colon cell line NCM460.

METHODS: Paracellular permeability of NCM460 monolayers was determined by transepithelial electrical resistance and dextran permeability. Expression of TJ proteins in NCM460 cell monolayers was detected by Western blotting and quantitative real-time polymerase chain reaction.

RESULTS: *L. plantarum* played an important role in increasing transepithelial electrical resistance and decreasing the permeability to macromolecules of NCM460 monolayers against the disruption caused by enteropathogenic *Escherichia coli* (*E. coli*) or enteroinvasive *E. coli*. *L. plantarum* also prevented the decrease in the expression of TJ proteins and F-actin in NCM460 cells.

CONCLUSION: *L. plantarum* can protect against dysfunction of NCM460 intestinal epithelial barrier caused by enteropathogenic *E. coli* or enteroinvasive *E. coli*, and thus can be a potential candidate of therapeutic agents for the treatment of intestinal diseases.

Key words: *Lactobacillus plantarum*; NCM460; Tight junction; Intestinal barrier

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INTRODUCTION

The human intestinal system included a group of viable microorganisms, which exceed the total number of somatic and germ cells[1-3]. Therefore, the human colon is confronted with the highest bacterial load in the digestive tract with enormous bacteria per gram of feces. Growing evidence showed that bacteria closely adherent to the mucosa are more relevant to human body, compared with those evacuated in the feces[4,5]. There is a homeostasis between probiotics and pathogens in the intestinal

systems of healthy individuals[6,7], which, if broken, may lead to an imbalanced ecological microenvironment and subsequent intestinal barrier dysfunction[8,9]. Thereafter, the accumulation of pathogens and secretory products, such as the exotoxins and secretory antigens, can also directly or indirectly initiate and amplify the local and systemic inflammatory responses[10,11]. Probiotics of the genus lactobacillus that reside in the human intestine play an important part in maintaining the homeostasis of gut flora by adhering and colonizing to the intestinal mucosa and competing with pathogenic bacteria, such as pathogenic *Escherichia coli* (*E. coli*)[12,13]. Enteric diseases with flora disequilibrium have been treated with lactobacillus over the past decades[14-16]. There is evidence indicating that the modulation of the gut flora by lactobacillus can improve the intestinal epithelial barrier function[17].

Adhesion of lactobacillus to the intestinal epithelium initially involves the activation of specific binding between bacterial ligands and their corresponding surface receptors on the intestinal cells of the host, following the non-specific physical interactions[18,19]. Generally, these ligands are adhesive molecules either existing on the surface layer of the bacteria or secreting from the mycelium of the bacteria. Furthermore, these ligands could interact with the corresponding receptors on the surface of the intestinal epithelial cells. Thereafter, these adhesins activate specific signal transduction pathways in both the bacteria and host cells. The interaction between lactobacillus and the intestinal epithelial cells can also block the adhesion of other pathogenic bacteria to the receptors of the intestinal epithelial cells, such as enteropathogenic *E. coli* (EPEC) and enteroinvasive *E. coli* (EIEC). As a widespread member of the genus lactobacillus, *Lactobacillus plantarum* (*L. plantarum*) is commonly found in many fermented food and anaerobic plant products. Our previous studies demonstrated that *L. plantarum* was able to prevent colonic damage caused by EIEC or inflammation *in vitro*, *in vivo* and in patients with acute pancreatitis[20-24]. The normal human colon cell line NCM460, which is derived from the normal human colon mucosal epithelium and expresses colonic epithelial cell-associated antigens such as cytokeratins and villin, has been applied exclusively in various intestinal research areas, including the infectious diseases[25-27].

Our previous studies indicated that *L. plantarum* exerted its therapeutic effects by adhering to the intestinal epithelial cells, restoring tight junction (TJ) structure and function, and reducing paracellular permeability. However, studies about the interaction between lactobacillus and the human intestine were limited in the cancer cell line and the animal models, and further researches based on the normal human intestinal cells are still needed. Therefore, our study aims to investigate the protective effects of *L. plantarum* against epithelial barrier dysfunction of the normal human colon cell line NCM460 caused by EIEC and EPEC.

MATERIALS AND METHODS

Bacterial strains and culture conditions

L. plantarum CGMCC 1258 (generously provided by Dr. Xiao-Min Hang, the Onlly Institute of Life Science, Shanghai Jiao Tong University, Shanghai, China) was inoculated in 5% fresh De Man, Rogosa and Sharpe broth at 37℃ for 24 h, harvested by centrifugation (3500 × *g*) at 4℃ for 20 min, and washed with 50 mL 0.01 mol/L phosphate buffered saline (PBS) (pH 7.4). The EIEC strain ATCC 43893 (O124:NM) and EPEC strain ATCC 43887 (O111:NM) (both from Shanghai Municipal Center for Disease Control and Prevention, Shanghai, China) were grown in static Dulbecco's modified eagle media (DMEM) at 37℃ for 24 h. Quantification of bacterial density was measured at 600 nm (Beckman DU-50 spectrophotometer) with the colony forming units.

NCM460 cells were purchased from INCELL Corporation (San Antonio, TX, USA) and cultured in M3 media supplemented with 10% FBS, 100 U/mL penicillin and 100 g/mL streptomycin at 37℃ in a 95% humidified atmosphere with 5% CO_2, as previously described[25].

Measurement of transepithelial electrical resistance in NCM460 cell monolayers

NCM460 cells were grown on filters (Millicell culture plate inserts; 0.4 μm pore size; 0.6 cm^2 surface area) at 37℃ in a 95% humidified atmosphere, with 5% CO_2. At full confluence (10-14 d) (i.e. a monolayer was formed), a transepithelial electrical resistance (TER) of > 450 Ω.cm^2 monolayer was achieved as measured using a voltmeter (Millicell-ERS; Millipore, MA, USA). The intestinal epithelial monolayers were treated with EIEC or EPEC in the presence or absence of *L. plantarum*. In infection groups, 100 μL EIEC ATCC43893 (O124:NM) and EPEC ATCC43887 (O111:NM) at 1.0 × 10^8/mL were, respectively, added to the apical side of the cell culture insert for rapid infection of the monolayer, with an inoculation ratio of EIEC/EPEC to NCM460 cells of 100:1, and the insert was placed in a 50-mL tube and centrifuged at 200 × *g* for 4 min. In *L. plantarum* groups, *L. plantarum* (100 μL of 1.0 × 10^8/mL) was added onto the monolayer of NCM460 cells simultaneously with the EIEC/EPEC infection. NCM460 cells cultured under the same conditions but without the infection of EIEC/EPEC, and addition of *L. plantarum* served as the control group. Two experiments were performed separately for EIEC and EPEC.

The integrity of the confluent polarized monolayers was verified by measuring TER at different time intervals. TER (Ω.cm^2 monolayer) = (Total resistance - Blank resistance) (Ω) × Area (cm^2 monolayer). Because TER values often vary among individual NCM460 cultures, the electrical resistance value was recorded for each monolayer before and after the treatment, and the percentage in the decrease of TER from the baseline (%TER) was calculated.

Determination of dextran permeability in NCM460 monolayers

DMEM (0.2 mL) containing conjugated dextran was added to the apical compartment of Transwells (Corning Costar Co., MA, USA), and 0.4 mL DMEM alone added to the basolateral compartment. After treatment as described above, samples (0.5 mL) collected from the basolateral

Table 1 Primers used for real-time polymerase chain reaction amplification for genes encoding tight junction proteins in NCM460 cells

Gene	Upstream primer	Downstream primer
Occluding	GCAGCTACTGGACTCTACG	ATGGGACTGTCAACTCTTTC
Claudin-1	GTGCCTTGATGGTGGTTG	TGTTGGGTAAGAGGTTGT
JAM-1	GATGTGCCTGTGGTGCTG	GCTCTGCCTTGAGATAAGAA
ZO-1	AAGAGTGAACCACGAGAC	TCCGTGCTATACATTGAG

compartment were placed into a 96-well plate (Corning Costar Co., MA, USA) and analyzed to determine their fluorescent intensity using the Odyssey infrared imaging system (LI-COR Biosciences, NE, USA) at a wavelength of 700 nm. Relative intensity (RI; the integrated intensities of treated samples relative to the integrated intensity of untreated samples) was calculated to indicate the effect of the treatment.

Western blotting for determining the distribution and expression of TJ proteins in NCM460 cell monolayers

For Western blotting, NCM460 cells were cultured and the monolayers were treated as described above, and the protein samples from NCM460 cells was prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the previous studies[20]. SDS-PAGE was performed using the standard laboratory techniques with a discontinuous gradient, 5% (w/v) stacking gel and a 10% (w/v) separating gel, in a Mini-PROTEAN Ⅱ (Bio-Rad Laboratories, CA, USA). Briefly, samples were mixed with loading buffer containing SDS and mercaptoethanol, boiled for 3 min, centrifuged, and loaded onto the SDS-PAGE gel for separation. Molecular weights of samples were determined by comparing mobility with known marker proteins. Gel was then transferred to PVDF membrane (Millipore, MA, USA) in a semidry electroblotter (Bio-Rad Laboratories) for 120 min at 100 V. The membrane was washed three times (20 min each) with PBS containing 0.1% Tween-20 (PBS-T buffer). After blocking overnight in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and 5% dry powdered milk, membranes were washed three times for 5 min each with TBS-T and incubated with corresponding primary antibodies against TJ proteins (claudin-1, occludin, JAM-1, and ZO-1) and a cell cytoskeleton element F-actin (all from Abcam, MA, USA) for 2 h at room temperature. After three washes with TBS-T, the membranes were incubated for 1 h with corresponding HRP-conjugated secondary antibodies. The membrane was washed three times (60 min each) with PBS-T buffer. The TJ proteins were tested using enhanced chemiluminescence (ECL kit; Pierce, IL, USA) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction for detecting the mRNA expression of TJ proteins in NCM460 cells

mRNA expression of TJ proteins, including claudin-1, occludin, JAM-1, and ZO-1, was determined by quantitative real-time polymerase chain reaction (RT-PCR). After the treatment as described above, total RNA was isolated from NCM460 cells using the Trizol reagent (Gibco Brl, USA)[28], followed by DNase I treatment. The quantity and quality of RNA were verified with the ratio of absorbance values at 260 and 280 nm, and by visualization of the bands on agarose gels. For each sample, 600 ng mRNA was used in reverse transcription reaction (iScript kit from BioRad Laboratories) according to the manufacturer's specifications. Further analysis of mRNA levels of each group was performed by RT-PCR with a light-cycling system (LightCycler; Roche Diagnostics GmbH, Mannheim, Germany). Sequences of the primers used are listed in Table 1. The mRNA expression level was described as the ratio of the mean reading of the experimental group over that of the control group for NCM460 cells.

RESULTS

L. plantarum prevented EIEC/EPEC-induced decrease of TER in NCM460 cells

TER in the NCM460 cell monolayers was decreased significantly in response to infection with EIEC/EPEC compared with uninfected control cells. However, decrease of TER induced by EIEC/EPEC was prevented by the simultaneous treatment of *L. plantarum* (Figure 1A and B).

L. plantarum inhibited increased macromolecular permeability of NCM460 cell monolayers in response to EIEC/EPEC

EIEC/EPEC had an obvious enhancing effect on permeability of NCM460 cell monolayers, as compared with the uninfected control cells. However, this effect was inhibited by the co-treatment of *L. plantarum* (Figure 2A and B).

L. plantarum prevented the decreased expression of TJ proteins and a cell cytoskeleton element F-actin detected by Western blotting

The expression of TJ proteins, including claudin-1, occludin, JAM-1 and ZO-1, and the cytoskeleton element F-actin, was decreased in NCM460 cells infected with EIEC or EPEC (Figure 3A and B) compared with the control cells, as detected by Western blotting of epithelial whole cell protein extracts of NCM460 cells ($P < 0.001$). However, after the pre-treatment of *L. plantarum*, the expression of TJ proteins and F-actin remained at similar levels to the control cells.

L. plantarum prevented the decreased expression of TJ proteins as detected by quantitative RT-PCR

mRNA expression of TJ proteins, including claudin-1, oc-

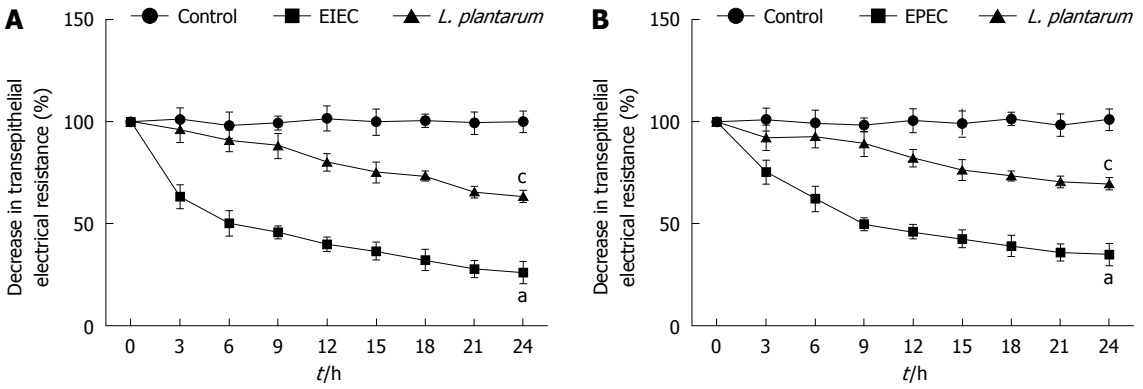

Figure 1 ***Lactobacillus plantarum*** **inhibited the decreased transepithelial electrical resistance through NCM460 cells induced by enteroinvasive** ***Escherichia coli*** **(A) or enteropathogenic** ***Escherichia coli*** **(B).** A: After infection with enteroinvasive *Escherichia coli* (EIEC) for 24 h, transepithelial electrical resistance (TER) of NCM460 monolayers was decreased significantly compared with the value in the control group. However, TER of EIEC-infected NCM460 monolayers, when simultaneously co-incubated with *Lactobacillus plantarum* (*L. plantarum*), was significantly higher than those in the EIEC-infected NCM460 monolayers; B: Similar results were obtained in the experiments with enteropathogenic *Escherichia coli* (EPEC). The data at each time point represent the mean ± SD obtained from four individual NCM460 monolayers. $^{a}P < 0.05$ *vs* control group; $^{c}P < 0.05$ *vs* corresponding EIEC or EPEC group.

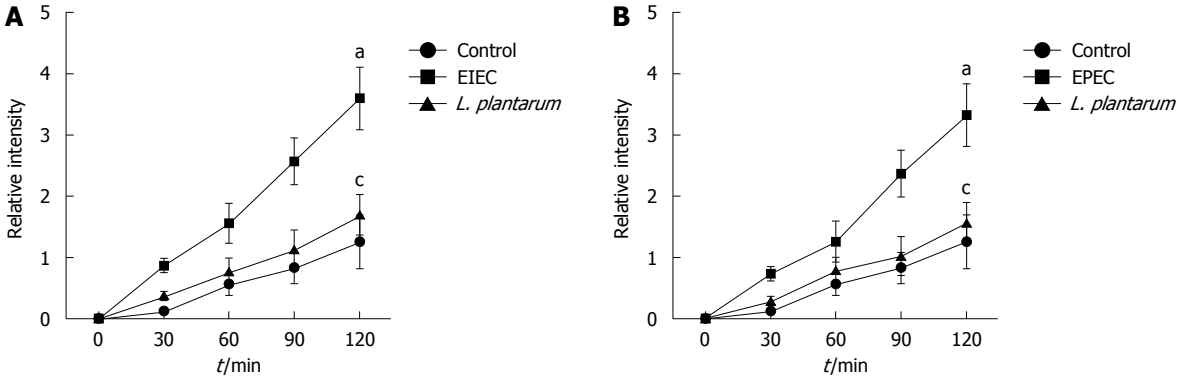

Figure 2 ***Lactobacillus plantarum*** **inhibited the increased macromolecular permeability through NCM460 cells induced by enteroinvasive** ***Escherichia coli*** **(A) or enteropathogenic** ***Escherichia coli*** **(B).** A: After infection with enteroinvasive *Escherichia coli* (EIEC) for 120 min, the relative intensity (RI) was significantly increased in the EIEC group compared with the control group. However, the RI was decreased significantly in *Lactobacillus plantarum* (*L. plantarum*) groups compared with the EIEC group; B: Similar results were obtained in the experiments with enteropathogenic *Escherichia coli* (EPEC). The data at each time point represent the mean ± SD obtained from four individual NCM460 monolayers. $^{a}P < 0.05$ *vs* control group; $^{c}P < 0.05$ *vs* corresponding EIEC or EPEC group.

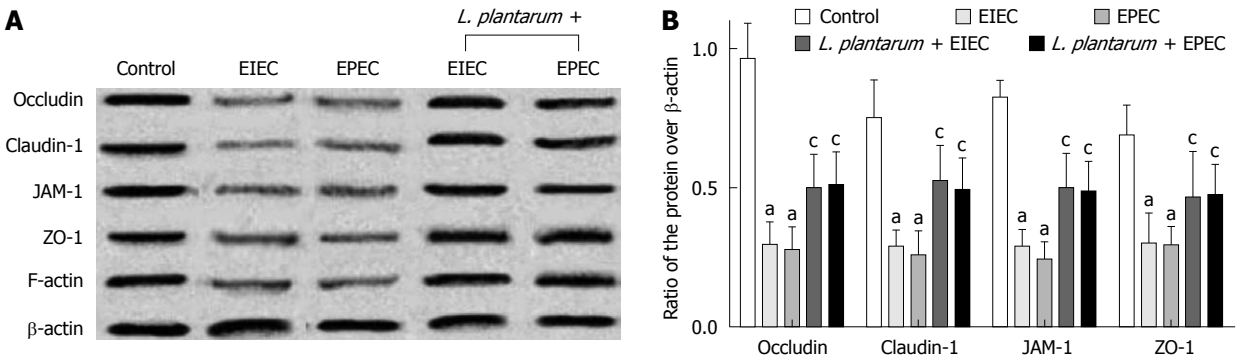

Figure 3 ***Lactobacillus plantarum*** **prevented the decrease in the expression of tight junction proteins in NCM460 cells induced by enteroinvasive** ***Escherichia coli*** **or enteropathogenic** ***Escherichia coli*** **detected by Western blotting.** A: The expression level of tight junction (TJ) proteins was high, including claudin-1, occludin, JAM-1 and ZO-1, in the control group. However, in the enteroinvasive *Escherichia coli* (EIEC) or enteropathogenic *Escherichia coli* (EPEC) group, TJ proteins were significantly decreased compared with the control group, which was not observed in the *Lactobacillus plantarum* (*L. plantarum*) group; B: Semi-quantitative analysis of Western blotting showed similar results. The data at each time point represent the mean ± SD obtained from four individual NCM460 monolayers. $^{a}P < 0.05$ *vs* control group; $^{c}P < 0.05$ *vs* corresponding EIEC or EPEC group.

cludin, JAM-1 and ZO-1, was significantly decreased in the NCM460 cells infected with EIEC or EPEC, as compared with the uninfected control cells (Figure 4A and B). However, treatment with *L. plantarum* raised mRNA expres-

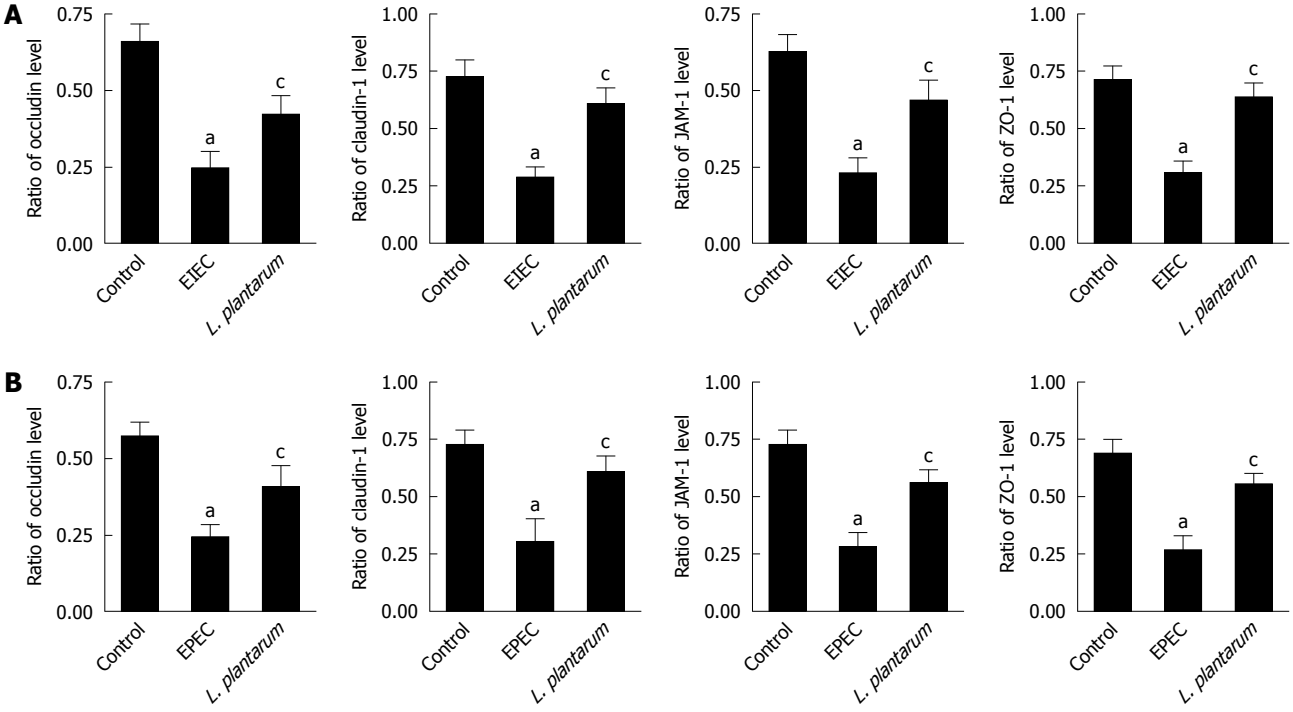

Figure 4 Protective effects of *Lactobacillus plantarum* and MIMP in mRNA expression of tight junction proteins in NCM460 cells detected by real-time polymerase chain reaction. A: The mRNA expression of tight junction (TJ) proteins, including occluding, claudin-1, JAM-1 and ZO-1, was decreased in enteroinvasive *Escherichia coli* (EIEC) group compared with the control group. However, in *Lactobacillus plantarum* (*L. plantarum*) group, the mRNA expression levels of the TJ proteins were similar to those in the control group; B: Similar results were obtained with enteropathogenic *Escherichia coli* (EPEC). The data represent the mean ± SD obtained from three individual NCM460 monolayers. $^{a}P < 0.05$ *vs* control group; $^{c}P < 0.05$ *vs* corresponding EIEC or EPEC group.

sion levels similar to those in the uninfected control cells (Figure 4A and B).

DISCUSSION

It has been reported that probiotics, such as *L. plantarum*, have beneficial effects on the human intestinal barrier function in patients with intestinal diseases[29]. Our previous studies also found that *L. plantarum* adhered to the intestinal epithelial cells, restored TJ structure and function, reduced paracellular permeability, and then showed the therapeutic effects[20,23]. However, the studies about the interaction between lactobacillus and the human intestine were only limited in the cancer cell line and the animal models. The present study investigated the protective effects of *L. plantarum* against epithelial barrier dysfunction of the normal human colon cell line NCM460.

L. plantarum played an important role in increasing TER and decreasing the permeability to macromolecules of NCM460 monolayers against the disruption caused by EIEC or EPEC. EIEC and EPEC had the ability to decrease TER and increase the permeability to macromolecules[20]. In the present study, we further observed that *L. plantarum* protected the epithelial barrier of NCM460 monolayers against the disruption caused by EIEC or EPEC. In other words, *L. plantarum* is able to attenuate the pathogen-induced decrease in TER, and inhibit the increase in the macromolecular permeability of dextran. Similar results were also found in other studies. Johnson-Henry reported that probiotics attenuated enterohemorrhagic *E. coli* O157:H7-induced drop in electrical resistance, and increased the corresponding intestinal barrier permeability[30].

Furthermore, we found that *L. plantarum* prevented the decrease in the expression of TJ proteins and F-actin in NCM460 cells. The expression of TJ proteins, including claudin-1, occludin, JAM-1 and ZO-1, and the cytoskeleton element F-actin were decreased in NCM460 cells infected with EIEC or EPEC compared with the control cells, as detected by Western blotting of epithelial whole cell protein extracts of NCM460 cells. However, after the pretreatment of *L. plantarum*, the expression of TJ proteins and F-actin remained at similar levels to the control cells. Other studies also found that *Lactobacillus rhamnosus* GG protected epithelial monolayers against EHEC-induced redistribution of the claudin-1 and ZO-1 TJ proteins. Resta-Lenert suggested that probiotics and/or commensals also reversed the epithelial damage produced by cytokines, and prevented the deleterious effects of tumor necrosis factor-α and interferon-γ in epithelial function[31].

Lactobacillus is reported to exert its beneficial effects by either producing bacteriostatic or bactericidal agents[32,33], competitively excluding pathogenic bacteria[34], or regulating immunomodulatory effects[13,31]. Furthermore, special signal transduction pathway is involved in the protective effects of *L. plantarum* on the intestinal epithelial barrier. Janus kinase inhibitor synergistically potentiated the effects of lactobacillus acidophilus on TER and permeability, while p38, ERK1, 2, or PI3K had no effects. After treated by lactobacillus, epithelial cells exposed to cytokines reduced the activation of SOCS3 and STAT1, 3.

We believe that our study broadens our knowledge of effects of *L. plantarum* in intestinal epithelial function and its therapeutic effects in the cellular and molecular mechanisms of intestinal barrier dysfunction and intestinal inflammation and justifies the use in inflammatory disorders, which is significant to both biotechnical and clinical fields. *L. plantarum* can protect against intestinal epithelial barrier dysfunction of NCM460 caused by EIEC or EPEC. However, the bacterial protein and its exact mechanisms of action remain unknown. We are conducting a study in an attempt to identify the protein and the smallest active domain within the protein from *L. plantarum* strain CGMCC1258 that is responsible for the adhesion of the bacterium to the intestinal epithelium. And further functional characterization by determining the effects of smallest active domain on the intestinal barrier function and immune responses is also in progress.

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COMMENTS

Background

Lactobacillus plantarum (*L. plantarum*) that resides in the human intestine plays an important part in maintaining the homeostasis of gut flora by adhering and colonizing to the intestinal mucosa and competing with pathogenic bacteria, which contributes to the protection of the human intestinal barrier function.

Research frontiers

Although *L. plantarum* exerted its therapeutic effects by adhering to the intestinal epithelial cells, restoring tight junction structure and function, and reducing paracellular permeability, the studies about interaction between lactobacillus and the human intestine were just limited in the cancer cell line and the animal models, and further studies based on the normal human intestinal cell had been unavailable.

Innovations and breakthroughs

Using the normal human colon cell line NCM460, this study investigated the protective effects of *L. plantarum* against epithelial barrier dysfunction caused by enteropathogenic *Escherichia coli* (*E. coli*) or enteroinvasive *E. coli*.

Applications

L. plantarum can be a potential candidate of therapeutic agents for the treatment of intestinal diseases.

Terminology

NCM460 cell line is a normal human colon cell line, which is derived from the normal human colon mucosal epithelium and expresses colonic epithelial cell-associated antigens such as cytokeratins and villin.

Peer review

This is a straightforward study extending previous work of the authors showing that *L. plantarum* maintains a high resistance to permeability to enteropathogenic and enteroinvasive *E. coli*. The work is extended to NCM460 colon cell cells in culture. Transepithelial electrical resistance was maintained high, dextran permeability was low and TJ protein expression was normal.

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BRIEF ARTICLE

Transarterial embolization ablation of hepatocellular carcinoma with a lipiodol-ethanol mixture

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Abstract

AIM: To determine the safety and effectiveness of transarterial embolization ablation (TEA) of hepatocellular carcinoma (HCC) with a lipiodol-ethanol mixture.

METHODS: Between January 1 and December 31, 2009, 15 patients with HCC (13 men/two women, aged 38-75 years) accepted TEA treatment and were enrolled in this study, including five newly diagnosed patients and 10 with refractory disease. Two months after TEA, angiography and contrast computed tomography (CT) were performed, and responses were assessed using a modified version of Response Evaluation Criteria in Solid Tumors (RECIST version 1.1). The follow-up period was to June 30, 2010.

RESULTS: Every new case was treated once. Angiography was performed immediately after TEA, and showed that the tumor-feeding vessels were completely embolized and that lipiodol was densely deposited inside tumors. Two months after treatment, contrast CT showed no enhanced lesions. Alpha fetoprotein levels returned to normal in four patients and markedly decreased in another. mean ± SD survival after treatment was 10.8 ± 4.5 mo. All five patients survived during the follow-up period. Ten patients with refractory disease were treated a total of 14 times. Angiography immediately after TEA showed that blood flow to the tumors was obviously decreased in all cases, and contrast CT showed obvious depositions of lipiodol. Two months after treatment, the tumors had shrunk (6/10) or were stable (3/10). One had progressed after 2 mo and died of tumor rupture 3 mo after TEA. mean ± SD survival after treatment was 8.6 ± 4.3 mo; two patients survived during the follow-up period. Adverse effects included reversible hepatic decompensation, upper abdominal pain, and fever.

CONCLUSION: TEA is an effective therapy for patients with HCC and might be more effective than transcather arterial chemoembolization for treating refractory disease.

Key words: Transarterial embolization ablation; Lipiodol-ethanol mixture; Hepatocellular carcinoma

Peer reviewer: Michael A Fink, MBBS, FRACS, Department of Surgery, The University of Melbourne, Austin Hospital, Melbourne, Victoria 3084, Australia

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common tumor worldwide and the third most common cause of tumor-related death[1-3]. HCC is not sensitive to radiotherapy or chemotherapy, therefore, surgery is still the treatment of choice. Unfortunately, < 30% of patients with HCC benefit from surgical resection because of unfavorable tumor location, stage or extent of disease, limited liver functional reserve, or high operative risk[4,5].

Minimal invasive ablation, a new treatment for HCC, achieves complete responses in > 80% of tumors < 3 cm in diameter. However, in tumors of 3-5 cm in diameter, the complete response rate is only 50%[6]. Therefore, ablation is not recommended for tumors > 5 cm. HCC is a hypervascular tumor that receives mostly hepatic arterial blood[7]. Since its introduction by Yamada *et al*[8,9] in the 1980s, transcatheter arterial chemoembolization (TACE) has been widely used to treat patients with inoperable liver tumors. The theoretical basis of TACE for treating HCC is that chemotherapeutic agents mixed with embolic material and injected into the hepatic artery will embolize the arteries that supply the tumor and kill the tumor cells[8,9]. However, Liu *et al*[7] also have reported that many HCC tumors, especially those > 5 cm in diameter, receive blood from both the hepatic artery and the portal vein. Therefore, TACE must be administered more than once, and tumor necrosis might still be incomplete[10-12].

Ethanol can kill tumor cells, and percutaneous ethanol injections have been used to treat unresectable small HCC tumors[13]. Ethanol can also produce permanent microcirculatory embolization that inhibits tumor growth by denaturing protein, coagulating platelets, and dehydrating vascular endothelial cells in tumors[14,15].

Transarterial embolization ablation (TEA) has been used to treat HCC with lipiodol-ethanol mixture (LEM)[16-18]. Compared with TACE, TEA has the following potential advantages: (1) LEM can permanently embolize tumor-feeding vessels that come from the hepatic artery and portal vein[15,17,19]; therefore, TEA might have a stronger antitumor effect than TACE; (2) after TACE, tumor and peritumoral normal tissues are ischemic, hypoxic, and secrete angiogenic substances that provide new conditions for tumor proliferation and recurrence[20,21], whereas TEA leads to infarction of the entire tumor and of the peritumoral normal liver tissue without collateral circulation; and (3) embolization of tumor vessels by LEM helps ethanol diffuse into the tumor[18,22-26]. It has been reported that the mean ± SD lipiodol retention rate (89.5% ± 10.7%) after TEA was significantly higher than after TACE (47.5% ± 21.2%) in liver lesions, and 1- and 2-year survival after TEA (93.3% and 80.0%, respectively) was also significantly higher than that after TACE (73.3% and 43.3%, respectively). Furthermore, the 1- and 2-year incidence of extrahepatic metastasis after TEA (both 0%) was substantially lower than it was after TACE (35.5% and 39.2%, respectively)[18].

In the present small pilot study, we sought to determine further the safety and effectiveness of TEA in treating patients with newly diagnosed and refractory HCC tumors.

MATERIALS AND METHODS

Patient recruitment

The protocol was approved by the Institutional Review Board of the Department of Medical Imaging and Interventional Radiology, Cancer Center, Sun Yat-sen University, Guangzhou, Guangdong, China. A total of 15 HCC patients who provided written informed consent before being enrolled in the study were treated with TEA from January 1 to December 31, 2009. Inclusion criteria included accurate proof of HCC, refusal of or contraindication to surgical resection (five cases), refractory disease (10 cases, progressed after TACE), Eastern Cooperative Oncology Group performance status not greater than 2. Exclusion criteria included total serum bilirubin ⩾ 50 μmol/L, serum albumin level < 28 g/L, and evidence of extrahepatic disease at presentation.

The demographic data of these patients are shown in Table 1. There were 13 men and two women with an average age of 55.7 ± 10.3 years (range: 38-75 years). All 15 patients were positive for hepatitis B and had concomitant cirrhosis. With regard to liver function status, 13 had Child-Pugh classification grade A and two had grade B. Of the 15, five had been recently diagnosed with single-nodule HCC (patients with new tumors and tumor diameter ⩽ 5 cm) and 10 patients with refractory tumors, including five with huge lesions (> 5 cm) and five with diffuse lesions. The five patients recently diagnosed with single-nodule HCC were all α fetoprotein (AFP)-positive and the lesion diameters ranged from 2.6 to 4.8 cm (mean ± SD, 3.7 ± 1.0 cm). The lesions were in the right lobule in two patients and in the left in three patients. Three of the five patients with huge HCC were AFP-positive, and all lesions were in the right lobule and the lesion diameters ranged from 8 to 15 cm (10.8 ± 3.1 cm). The five patients with diffuse lesions were all AFP-positive.

Treatment protocol

After puncturing the right femoral artery with the Seldinger technique, a Yashiro (5F, Terumo Heart, Inc., Japan) or R-H (5F, Terumo Heart) catheter was inserted into the common hepatic or superior mesenteric artery through a 5-F catheter sheath. Angiography was then performed to evaluate tumor blood supply and the surrounding vascular anatomy. Next, a 3F microcatheter (Terumo Heart) was inserted into the catheter (which remained in the proximal hepatic artery) and was advanced into the tumor-feeding arteries. For diffuse tumors, the microcatheter was inserted into segmental or subsegmental liver arteries to avoid the gastroduodenal and cystic arteries.

One percent lidocaine (from 2% lidocaine with saline, 1:1 by volume), 5-10 mL, was injected through a 2.5-mL syringe and followed by injection of a mixture of lipiodol (Guerbert S.A., Villepinte, France) and absolute ethanol (Department of Pharmacy, General Hospital of Guangzhou Military Command, China), 1:1 by volume. The in-

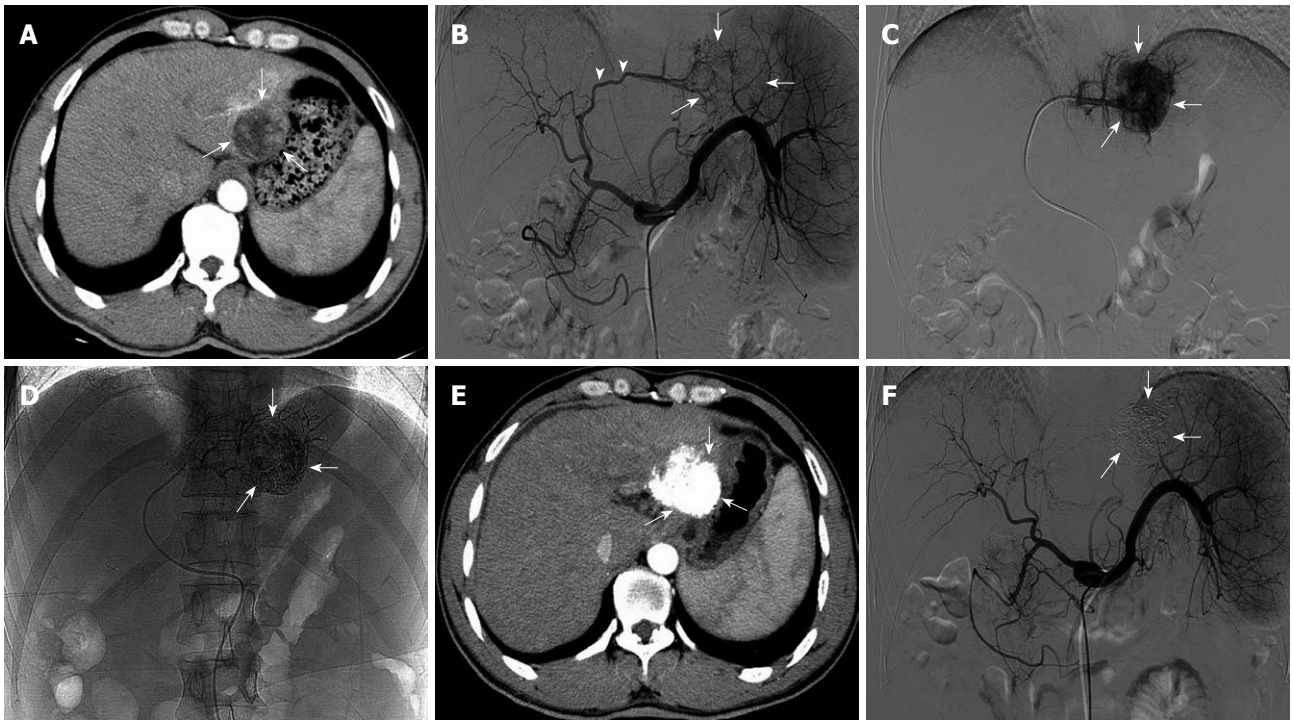

Figure 1 A newly diagnosed patient with single-nodule treated by transarterial embolization ablation. A: An enhanced computed tomography (CT) scan of hepatocellular carcinoma tumor before treatment. The tumor measured 3.5 cm × 3.3 cm (arrows); B: Hepatic artery angiography showed the thick blood vessels of the tumor (arrowheads) and the abnormal vascular group (arrows) before treatment; C: Superselective angiography clearly showed tumor staining (arrows); D: After transarterial embolization ablation with an lipiodol-ethanol mixture, lipiodol accumulated in the tumor (arrows); E: An enhanced CT scan 2 mo after treatment showed dense deposition of lipiodol in the tumor without enhancement (arrows); F: Hepatic artery angiography showed the absence of tumor blood vessels and tumor staining at 2 mo after treatment (arrows).

Table 1 Demographic data

	n
Features	
Sex (M/F)	13/2
Age (yr)	55.7 ± 10.3
Seropositive for HBsAg/HCV	15/0
Underlying cirrhosis (+/-)	13/2
Radiological/histological evidence of HCC	14/1
Newly diagnosed/refractory cases	5/10
Serum AFP level (μg/L)	
≤ 200	2
> 200	13
Liver function status	
Grade A/B	13/2
Feature of tumors	
Single nodule[1] (≤ 5 cm)	5
Huge lesion[2] (> 5 cm)	5
Diffused lesion[3]	5

[1]Patient with one tumor that was ≤ 5 cm in diameter; [2]Patient with one tumor that was > 5 cm in diameter; [3]Patient with multiple different size tumors. HBsAg: Hepatitis B surface antigen; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; AFP: α fetoprotein.

jection of LEM was stopped when the tumor-feeding arteries were occluded, or when injection reflux was noted, or when the amount injected reached 60 mL.

Follow-up was to June 30, 2010 and measurements consisted of the degree of tumor embolization as determined visually immediately after TEA by angiography, judged by two skilled radiologists, liver function [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] for 1-7 d after TEA, and AFP levels, computed tomography (CT) or magnetic resonance imaging, and changes of postoperative symptoms and signs up to 8 wk after treatment. Responses were assessed using a modified version of Response Evaluation Criteria in Solid Tumors (RECIST version 1.1)[27].

RESULTS

Every newly diagnosed patient was treated once. The maximum total dose of LEM was 54 mL (10.8 ± 5.4 mL). Angiography immediately after TEA showed that all tumor-feeding arteries were completely embolized and that lipiodol had been densely deposited inside tumors. CT images 2 mo after treatment showed lesions with non-enhancement that had been replaced by deposition of lipiodol (Figure 1). AFP levels returned to normal in four patients (before TEA: 2947.6 ± 3724.5 μg/mL; post-TEA: 13.7 ± 8.2 μg/mL; $P = 0.04$) and decreased substantially in another patient (from 37 625 μg/mL to 26 376 μg/mL). Mean survival after treatment was 10.8 ± 4.5 mo. All five patients survived during the follow-up period (Table 2).

All refractory patients were treated a total of 14 times. The total dose of LEM was 256 mL (mean: 18.3 ± 13.5 mL). Angiography immediately after TEA showed that blood flow to the tumors was obviously decreased. Two months after treatment, CT showed that lipiodol was deposited more obviously than before and the tumors were smaller

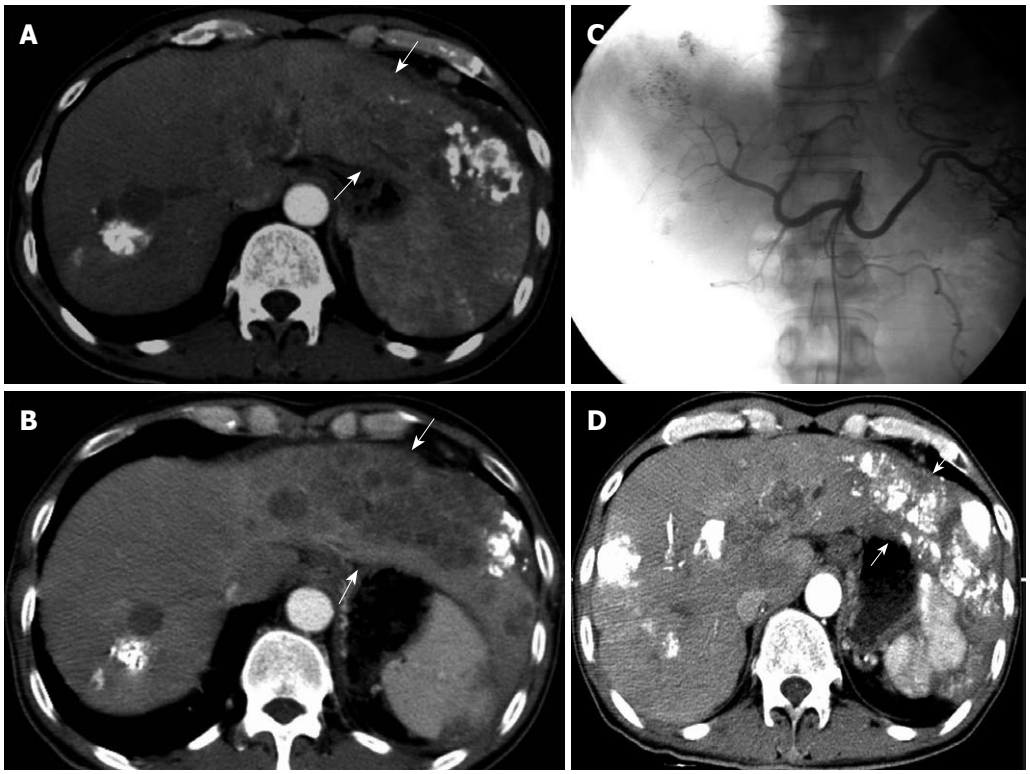

Figure 2 A patient with refractory tumors treated by transarterial embolization ablation. A: Limited lipiodol accumulation in a patient with a diffuse hepatocellular carcinoma tumor after transcatheter arterial chemoembolization (TACE) (arrows); B: After a second treatment with TACE, the tumor had progressed and showed less lipiodol accumulation (arrows); C: Hepatic artery angiography showed that tumor staining was not obvious. According to computed tomography images before transarterial embolization ablation (TEA), lipiodol-ethanol mixture was injected through the left and right hepatic arteries; D: Two months after TEA, the tumor had shrunk and lipiodol accumulation had increased (arrows).

Table 2 Clinical efficiency of newly diagnosed case

Newly diagnosed cases	Tumor response evaluation[1]		Follow-up period (mo)	Survival[2]
	Target lesion 2 mo post-TEA	Time to progress (mo)		
1	CR	9.9	16.8	Yes
2	CR	6.1	14.1	Yes
3	PR	6.3	9.5	Yes
4	PR	5.1	7.5	Yes
5	PR	0	6.3	Yes

[1]RECIST version 1.1; [2]Survival (yes), death (no). TEA: Transarterial embolization ablation; CR: Complete response; PR: Partial response.

(6/10) or stable (3/10) in nine patients (Figure 2). For eight AFP-positive patients, AFP levels returned to normal in one patient, were decreased in six after a mean 2.5 ± 1.1 mo, and continued to rise in one. The mean survival was 8.6 ± 4.3 mo after treatment. Two patients survived during the follow-up period (Table 3).

Adverse events

Adverse events post-TEA are listed in Table 4. Of the 15 patients, 10 had transient elevations in transaminase after TEA, all of whom returned to normal after drug treatment to protect liver function. All patients had intraoperative or postoperative upper abdominal pain, which was relieved or eliminated after symptomatic treatment. Eleven patients had fever at 2-7 d after TEA but recovered with the administration of antipyretic drugs or dexamethasone (5-10 mg). There were no other serious adverse effects.

Table 3 Clinical efficiency of refractory cases

Refractory cases	Tumor response evaluation[1]		Follow-up period (mo)	Survival[2]
	Target lesion[4] 2 mo post-TEA	Time to progress (mo)		
1	PR	6.5	8.1	No
2	PR	7.8	14.3	Yes
3	PR	4.2	7.1	No
4	SD	3.9	6.1	No
5	SD	3.7	4.9	No
6	PR	6.2	10.1	No
7	SD	3.0	5.1	No
8	PR	6.5	16.7	Yes
9	PR	5.0	9.9	No
10	PD	[3]	3.5	No

[1]RECIST version 1.1; [2]Survival (yes), death (no); [3]Continued to progress; [4]Percentage of response evaluation: CR (0%), PR (60%), SD (30%), PD (10%). TEA: Transarterial embolization ablation; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

DISCUSSION

Currently, TACE has become an important palliative treatment for patients with inoperable HCC[5,11,12,28-30]. Embolization of tumor-feeding arteries also prolongs the exposure and concentration of tumor drugs and chemo-

Table 4 Adverse events

Adverse events	*n*
Pain	15
Fever	11
Hepatic decompensation	10
Liver abscess	0
Biliary stricture or obstruction	0

therapeutic agents in the tumor microvascular bed, rather than allowing the drugs to reach the systemic circulation, which would reduce their effectiveness[5,8,9]. However, HCC tumors are occasionally partially supplied by the portal vein, especially when capsule formation around the tumor is incomplete[7,17,31]. After TACE, tumor blood supply from the portal vein increases, which makes achieving complete tumor necrosis difficult. In addition, embolization after TACE creates ischemia and hypoxia in the tumor and surrounding normal tissue, which leads tumor cells and peritumoral normal liver cells to secrete angiogenic factors, such as vascular endothelial growth factor and fibroblast growth factors, which promote collateral circulation and restoration of the tumor blood supply, which leads to tumor proliferation and recurrence[20,21]. To achieve complete tumor necrosis, the blood supply to the tumor from the liver artery and portal vein and fistula between them needs to be stopped, and the formation of collateral circulation needs to be prevented[32,33].

In animal experiments, Kan *et al*[15] have proved that transarterial injection of LEM achieves lobar ablation effects because it simultaneously embolizes the hepatic artery and portal vein. Ethanol is a strong protein coagulant that can inactivate tumor cells directly *in situ* and cause vascular endothelial injury and platelet cohesion, which results in permanent occlusion of tumor-feeding vessels and tumor infarction without causing collateral circulation[14,15].

In 1993, Matsui *et al*[17] described transarterial ethanol embolization of HCC. However, ethanol is not radiopaque, and its flow and speed are difficult to visualize when it is administered. In contrast, LEM traces not only the ethanol, which is necessary to avoid regurgitation and ectopic embolization, but also enhances the effects of embolization. Therefore, we call the transarterial injection of LEM TEA to distinguish it from TACE by injection of gelatin sponge or other embolic materials.

Optimal lipiodol:ethanol ratios

We treated 15 HCC patients with a mixture of equal volumes of lipiodol and ethanol. The results showed that TEA could completely embolize tumor-feeding vessels, reduce the size of single-nodule HCC tumors, and decrease levels of AFP. Enhanced CT examinations of the liver showed no enhancement of lesions. For refractory HCC, TEA can also shrink tumors and decrease AFP levels.

Reports were varied about the effectiveness of different ratios of lipiodol to ethanol. Matsui *et al*[17] have treated 100 patients with 124 HCC tumors < 4 cm with equal volumes of lipiodol and ethanol through hepatic artery injection. Survival rates for 82 patients with Child class A or B disease at 1 and 4 years were 100% and 67%, respectively. Kan *et al*[15] have reported that embolic effects were better when the lipiodol:ethanol ratio was 5:1, 4:1, or 3:1 than if the ratio was 1:1. However, several studies have reported good results in treating HCC with transarterial injections with lipiodol:ethanol ratios between 3:1 and about 1:3[16,17,19,34-36].

We think that the lipiodol:ethanol ratio can be adjusted according to the type of HCC. For single-nodule HCC, a relatively high lipiodol ratio (such as 2-3:1) is appropriate because the tumors are relatively small, have a complete capsule, and have a blood supply that comes mainly from the hepatic artery, with few fistulae between the hepatic artery and the portal vein. The injury to small arteries is relatively slight because of the lower percentage of ethanol in the mixture, and accordingly, more LEM is deposited inside the tumor. Meanwhile, the uptake of lipiodol by liver cancer cells means that the mixture is retained longer in tumor cells and has more time to produce antitumor effects[15,37].

For refractory HCC, a relatively high ethanol ratio is appropriate to inhibit the double blood supply from the hepatic artery and the portal vein with fistulae between them. Ethanol can permanently embolize tumor-feeding vessels and block the fistulae, as well as reducing the loss of lipiodol and extending the exposure time in the tumor cells, which achieves a better therapeutic effect. However, further research is needed to determine the optimal lipiodol:ethanol ratios for different types of HCC.

Treating adverse events

The most common adverse events after TEA were liver dysfunction, fever, and upper abdominal pain. In this study, 10 of 15 patients experienced liver dysfunction, and mean levels of ALT and AST rose to 600-1200 U/L in serious cases; therefore, liver function needs to be monitored closely and protected after TEA. We usually review patient's liver function the day after TEA and then every 2-3 d until it returns to normal or nearly normal. Antipyretic drugs or dexamethasone are usually effective in treating fever. Damage to the vascular wall caused by ethanol results in upper abdominal pain in all patients. We administer intramuscular injections of 10 mg diazepam at 10-30 min before treatment and 10 mg intramuscular morphine after catheterization of tumor-feeding arteries. We also inject 5-10 mL 1% lidocaine into the arteries before LEM. Patients generally tolerate TEA given this preparation. We stop injection of LEM for 1-2 min if patients feel pain and resume injection when the pain is relieved after injection of lidocaine through the catheter. For those who still feel pain after TEA, a fentanyl patch (4.2 mg from beginning) can be applied.

Potential problems and study limitations

Ethanol is a strong protein coagulant and a permanent embolic material, but it can also injure normal liver tissue and vessels[4,16,27,35,36,38]. Therefore, tumor blood vessels must be selected and treated carefully to avoid severe liver damage,

and even failure, and other serious complications caused by shallow intubation. On the other hand, excessively deep intubation can lead to incomplete embolization.

In addition, the speed of injection can also affect the efficacy of TEA. Blood dilutes ethanol and therefore will weaken the effect of ethanol on tumor cells if it is injected slowly, yet rapid injection might destroy blood vessel walls and form a vessel-casting mold. Injection of too much mixture can lead to reflux into normal liver tissues or to the blood vessels that supply the gastrointestinal tract. Therefore, research is needed into the effect of injection speed and on the dose of LEM.

Our study had some limitations. The sample size was small, but it was large enough to prove the concept. The follow-up after TEA was also short, and long-term efficacy needs to be studied further. In addition, some huge tumors had some necrosis before TEA, and the degree of tumor shrinkage was difficult to determine. Finally, we determined efficacy only on the basis of angiographic and CT imaging, without a postoperative pathological examination. These limitations can easily be avoided in larger, longer, and better-funded studies.

On the basis of our findings, we conclude that TEA is effective therapy for patients with HCC and might be better than TACE for treating refractory disease. Further studies, including randomized controlled trials, are warranted to confirm its role.

COMMENTS

Background

Transarterial embolization ablation (TEA) is a new treatment strategy for hepatocellular carcinoma (HCC). Some researchers have reported its effectiveness in HCC, especially in single-nodule HCC. However, its clinical effect for refractory HCC [not suitable for surgery and local ablation and with disease progression after transcatheter arterial chemoemblization (TACE)] remains unknown.

Research frontiers

TACE is accepted widely in treating advanced HCC because of its confirmed clinical effect. Advanced HCC tumors often receive blood from the hepatic artery and portal vein. Therefore, TACE must be administered more than once, and tumor necrosis can still be incomplete. In the present study, the authors demonstrated that TEA with lipiodol-ethanol mixture was an effective therapy for patients with HCC and might be more effective than TACE for treating refractory disease.

Innovations and breakthroughs

TACE with a mixture of lipiodol and ethanol has been shown to be an effective treatment for intrahepatic lesions of HCC, although it has not been widely used or described. Recent reports have highlighted the importance of this new method for its good results in nodular or capsulated HCC. Furthermore, this study suggests that this new method can also be applied in advanced refractory HCC.

Applications

Due to the exciting results and acceptable adverse events, TEA could represent a future treatment strategy for advanced HCC.

Terminology

Lipiodol is a very important embolization agent in TACE of HCC. Ethanol can kill tumor cells, and percutaneous ethanol injections have been used to treat unresectable small HCC tumors (chemical ablation). Ethanol can also produce permanent microcirculatory embolization that inhibits tumor growth by denaturing proteins, coagulating platelets, and dehydrating vascular endothelial cells in tumors. Transarterial lipiodol-ethanol mixture injection is called TEA.

Peer review

The authors report on the prospective follow-up of 15 patients treated with lipiodol-ethanol injection. The results are interesting. They reveal that TEA is a effective and safe treatment for advanced HCC. A further comparative study with TACE is needed to re-evaluate this new method.

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BRIEF ARTICLE

Mitochondrial uncoupling protein 2 expression in colon cancer and its clinical significance

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Abstract

AIM: To detect the expression of mitochondrial uncoupling protein 2 (UCP2) in colon cancer and analyze the relation between UCP2 expression and clinical pathological features of colon cancer.

METHODS: Fifteen colon tissue samples and 15 its adjacent tissue samples were obtained from colon cancer patients during surgical interventions. UCP2 expression was detected with immunohistochemical method in 10 normal controls, 10 hyperplastic polyp patients, 20 tubular adenoma patients and 78 colon cancer patients. Patients with rectal cancer were excluded. Quantitative reverse transcription polymerase chain reaction and Western blotting were used to detect UCP2 expressions in colon cancer tissue samples and its adjacent tissue samples. Relation between UCP2 expression and clinical pathological features of colon cancer was also analyzed.

RESULTS: The UCP2 mRNA expression level was four-fold higher in colon cancer tissue samples than in its adjacent tissue samples. The UCP2 protein expression level was three-fold higher in colon cancer tissue samples than in its adjacent normal tissue samples. The UCP2 was mainly expressed in cytoplasm. The UCP2 was not expressed in normal colon mucosa. Strong positive staining for UCP2 with a diffuse distribution pattern was identified throughout the mucosa in colon cancer tissue samples with a positive expression rate of 85.9%. The UCP2 expression level was higher in colon cancer tissue samples at clinical stages Ⅲ and Ⅳ than in those at stage Ⅰ + Ⅱ. Univariate analysis showed that the high UCP2 expression level was significantly correlated to colon cancer metastasis (hazard ratio = 4.321, confidence interval = 0.035-0.682, P = 0.046).

CONCLUSION: UCP2 is highly expressed in human colon cancer tissue and may be involved in colon cancer metastasis.

Key words: Mitochondrial uncoupling protein 2; Colon cancer; Uncoupling protein 2; Clinicopathologic characteristics

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INTRODUCTION

Colon cancer is one of the malignant tumors threatening to human health, and the mortality of colon cancer patients ranks second among all malignant tumors in developed countries. In recent years, its incidence has been increas-

ing in China, particularly in the economically developed coastal cities[1,2]. Its etiology and pathogenesis remain unclear. Great achievements have been made in studies on changes in tumor suppressor gene or proto-oncogene, but some problems cannot be explained. Recent studies showed that mitochondrial dysfunction is involved in the occurrence and development of tumor[3-5]. Uncoupling protein-2 (UCP2) is a mitochondrial membrane protein, which negatively regulates the production of reactive oxygen species (ROS)[6-8]. Adaptive mechanisms of cancer cells include resistance to tumor growth inhibition and evasion of apoptosis, and cellular events that are appreciably affected by oxidative stress[9,10]. The UCP2 expression level is significantly higher in colon cancer tissue than in its adjacent tissue and UCP2 may play a role in intestinal epithelial cells from benign to malignant transformation[11]. However, the role of UCP2 in development of colon cancer is unclear. In this study, the expression of UCP2 in normal human colon tissue and colon cancer tissue was detected, and the relation between UCP2 expression in colon cancer tissue and clinical pathological features of colon cancer was also analyzed.

Table 1 Clinical and histological features of colon cancer patients

Characteristics	*n* (%)
Gender	
Female	33 (42.3)
Male	45 (57.7)
Age (yr): median 60.7, range 31-78	
< 60	36 (46.2)
⩾ 60	42 (53.8)
Primary sites	
Left colon	44 (56.4)
Right colon	34 (43.6)
Clinical stage	
Ⅰ (T1N0M0)	10
Ⅱ (T2N0M0)	17
Ⅲ	37
Ⅳ	14
Tumor differentiation	
Well	16
Moderately	38
Poorly	24

MATERIALS AND METHODS

Patients and tissue samples

Fifteen colon caner tissue samples and 15 its adjacent tissue samples were obtained from the First Affiliated Hospital of Nanjing Medical University, snap-frozen and stored at -70℃. UCP2 expression was detected with immunohistochemical method in 10 normal controls, 10 hyperplastic polyp patients, 20 tubular adenoma patients, and 78 patients (45 males and 33 females) with colon cancer at different stages. Rectal cancer patients were excluded. Clinical pathological characteristics of the 78 colon cancer patients are listed in Table 1.

Immunohistochemistry

Tissue sections were stained with rabbit polyclonal antibody against human UCP2 (LS-C41270, LifeSpan BioSciences, Seattle, WA, USA), horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Inc, USA) and visualized using peroxidase. Negative control sections were treated in PBS instead of primary antibodies. Intensity of UCP2 staining was scored as negative (0), weak (+1), moderate (+2), and strong (+3).

Quantitative reverse transcription polymerase chain reaction

After extraction of total RNA from snap-frozen colonic surgical samples with TRIzol reagent (Invitrogen, Carlsbad, CA) and removal of contaminating genomic DNA with DNase I and RNasefree (Roche Diagnostics Corp., Indianapolis, IN), reverse transcription polymerase chain reaction (RT-PCR) was performed using a first-strand cDNA synthesis kit (Roche Diagnostics Corp, Indianapolis, IN) following its manufacturer's instructions. Quantitative RT-PCR was performed using an ABI Prism 7300 real-time PCR detection system (Bio-Rad, Hercules, CA) following its manufacturer's instructions. The sequences of UCP2 and internal control GAPDH primers used in this study are as follows: Ucp2 gene: R: 5'-TCAGAATGGTGCCCATCACA-3', F: 5'-CCGGTTACAGATCCAAGGAGAA-3', GAPDH: R: 5'-ACCCTGTTGCTGTAGCCA-3', F: 5'-CCACTCCTCCACCTTTGAC-3'. The PCR amplification conditions were 95℃ for 5 min, followed by 40 cycles at 95℃ for 30 s, at 60℃ for 1 min, and a final extension at 72℃ for 3 min. The relative amount of gene expression = 2 Δct × 100 (Δct = ct target gene-ct internal reference).

Western blotting for UCP2

Sample was prepared for Western blotting using a mitochondria isolation kit following its manufacturer's instructions (AR0156, Wuhan Boster Biological Technology, LTD, Wuhan, China). An equal amount of protein was size-fractionated with 12.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Perkin-Elmer Life Sciences, Boston, MA). Immunoblots were developed using anti-UCP2 antibody (C-20; Santa Cruz Biotechnology, Inc, USA). Mitochondrial protein obtained from wild-type mouse spleen was used as a positive control. An equal loading was confirmed using cytochrome C (sc-13561, Santa Cruz Biotechnology, Inc, USA). Membranes were incubated with secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc, USA), detected with enhanced chemiluminescence (ECL detection system, NEN, Boston, MA, USA), and used for visualization.

Statistical analysis

Data are expressed as mean ± SE. Correlation between categorical groups was evaluated by χ^2 test or Fisher's exact test, when appropriate. Univariate analysis of hazard model was employed to detect independent predictors of colon cancer metastasis. When appropriate, linear regression and Pearson r correlation were calculated. Two-tailed *P* value of ⩽ 0.05 was considered statistically significant.

Table 2 Uncoupling protein 2 mRNA expression in human colon cancer and peritumoral tissue samples detected by quantitative real-time polymerase chain reaction

Patient No.	Tissue	UCP2 mRNA relative abundance	T/P
1	T	8.12	3.45
	P	2.35	
2	T	19.68	5.70
	P	3.45	
3	T	13.6	2.96
	P	4.6	
4	T	21.4	2.32
	P	9.21	
5	T	26.2	3.67
	P	12.7	
6	T	78.6	2.51
	P	31.2	
7	T	86.5	3.59
	P	24.1	
8	T	12.31	6.00
	P	20.5	
9	T	45.2	8.85
	P	5.1	
10	T	36.1	0.84
	P	42.6	
11	T	9.12	2.37
	P	3.85	
12	T	3.67	1.78
	P	2.05	
13	T	10.34	4.40
	P	2.35	
14	T	17.63	5.86
	P	3.01	
15	T	6.54	4.47
	P	1.46	
Average T/P ratio			3.92

UCP2: Uncoupling protein 2; T: Tumour; P: Peritumoral.

Table 3 Uncoupling protein 2 protein expression in human colon cancer and peritumoral tissue samples detected by Western blotting

Patient No.	Tissue	UCP2 protein (total gray)	T/P
1	T	2.10	2.14
	P	0.98	
2	T	4.16	5.33
	P	0.78	
3	T	2.45	2.82
	P	0.87	
4	T	5.14	4.25
	P	1.21	
5	T	3.02	3.70
	P	0.82	
6	T	6.24	2.70
	P	2.31	
7	T	5.55	5.00
	P	1.09	
8	T	8.27	3.93
	P	2.1	
9	T	9.43	8.65
	P	1.08	
10	T	1.96	0.78
	P	2.51	
11	T	3.15	3.80
	P	0.83	
12	T	7.12	2.13
	P	3.34	
13	T	4.51	3.72
	P	1.21	
14	T	7.12	5.45
	P	1.31	
15	T	2.54	4.17
	P	0.62	
Average T/P			3.27

UCP2: Uncoupling protein 2; T: Tumour; P: Peritumoral.

RESULTS

Expression of UCP2 mRNA in colon cancer and its adjacent tissue samples

The expression of UCP2 mRNA in 15 colon cancer tissue samples and 15 its adjacent tissue samples was detected by quantitative RT-PCR. The expression level of UCP2 mRNA was about four-fold higher in colon cancer tissue samples than in its adjacent tissue samples (Table 2).

Expressions of UCP2 protein in colon cancer and its adjacent tissue samples

The expression of UCP2 protein in 15 colon cancer tissue samples and 15 its adjacent tissue samples was detected by Western blotting. The expression level of UCP2 protein was three-fold higher in colon cancer tissue samples than in its adjacent tissue samples (Figure 1, Table 3).

Correlation between UCP2 protein and mRNA expressions in human colon cancer tissue samples

The correlation between UCP2 protein and UCP2 mRNA expressions in human colon cancer tissue samples was observed. A strong linear correlation was found between the T/P ratio of UCP2 mRNA and protein expression ($r = 0.7442$, $P < 0.05$), suggesting that increased UCP2 expression in colon cancer tissue is largely determined at the transcriptional level (Figure 2).

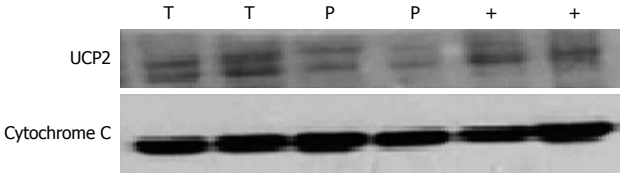

Figure 1 Western blotting analysis showing uncoupling protein 2 expression in human colon cancer tissue samples. T: Colon cancer tissue sample, P: Peritumor tissue sample; +: Positive control, spleen from wild type mouse. Cytochrome C was used as a loading control. UCP2: Uncoupling protein 2.

UCP2 expression in colon tissue samples detected by immunohistochemistry

The expression of UCP2 was detected by immunohistochemistry in colon tissue samples from 78 colon cancer patients, 20 adenoma patients, 10 hyperplastic polyp patients and 10 normal controls. UCP2 was mainly expressed in cytoplasm but not expressed in epithelium of normal colon. In contrast, strongly positive staining for UCP2 with a diffuse distribution pattern was identified throughout the mucosa in most tubular adenomas and adenocarcinomas. The positive staining rate was 85.9% and 55% for

Table 4 Uncoupling protein 2 expression in different diseases detected by immunohistochemistry

Tissue	*n*	Negative	Mild	Moderate	Strong	Rate of positive (%)
Normal colon	10	10	0	0	0	0
Hyperplastic polyps	10	9	2	0	0	20
adenoma	20	9	3	6	2	55
Colon cancer	78	10	15	30	22	85.9

Positive cells < 15% in negative control and ⩾ 15% positive cells were counted as positive. Staining intensity was classified as negative (0), mildly positive (+1), moderately positive (+2), and strongly positive (+3).

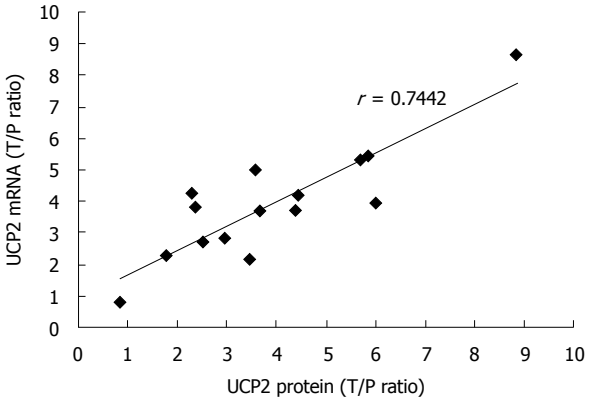

Figure 2 Correlation between uncoupling protein 2 and uncoupling protein 2 mRNA expressions in human colon cancer. UCP2: Uncoupling protein 2; T: Tumour; P: Peritumoral.

colon cancer tissue and colon adenoma tissue, respectively (Figure 3, Table 4).

Correlation between UCP2 expression and clinical pathological features of colon cancer

The correlation between UCP2 expression and clinical pathological features of colon cancer (including tumor stage and cell differentiation) was analyzed. The expression level of UCP2 was significantly higher in patients with colon cancer at clinical stages Ⅲ and Ⅳ than in those at stage Ⅰ + Ⅱ. However, the UCP2 expression seemed to be irrelevant to cell differentiation status, indicating that further study is needed (Table 5).

Analysis of colon cancer metastasis- related factors

The relation of colon cancer metastasis with sex and age of patients, tumor location, UCP2 expression and other features was assessed by univariate analysis, showing that cell differentiation and UCP2 expression were involved in colon cancer metastasis. However, colon cancer metastasis seemed to be irrelevant to age and sex of patients and tumor location (Table 6).

DISCUSSION

In this study, different UCP2 expressions in colon cancer tissue and normal peritumoral tissue samples were observed. The expression of UCP2 mRNA in colonic mucosa samples from patients undergoing colon cancer resection was detected by quantitative PCR. As shown in Table 2, the UCP2 mRNA expression level was 4-fold higher in colon cancer mucosal tissue samples than in grossly normal peritumoral colonic mucosal

Table 5 Relation between uncoupling protein 2 expression and clinical pathological features of colon cancer

Tumor differentiation/ clinical stage	Positive staining rate of UCP2 (%)	*P*
Tumor differentiation		
Well differentiation	72.8	0.072
Poor differentiation	91.4	
Clinical stage		
Stage Ⅰ + Ⅱ	65.1	0.032[1], 0.0248[2]
Stage Ⅲ	80.4	0.248[3]
Stage Ⅳ	92.6	

[1]*P* = 0.032, uncoupling protein 2 (UCP2) expression in colon tissue from patients with clinical stage Ⅲ *vs* that in colon tissue from patients with clinical stage Ⅰ + Ⅱ; [2]*P* = 0.0248, UCP2 expression in colon tissue from patients with clinical stage Ⅳ *vs* that in colon tissue from patients with clinical stage Ⅰ + Ⅱ; [3]*P* = 0.248, UCP2 expression in patients with clinical stage Ⅳ *vs* that in colon tissue from patients with stage Ⅲ.

Table 6 Univariate Cox proportional hazards analysis of metastasis variables in colon cancer patients

Variable	Hazard ratio	95% CI	*P*-value
Age (⩾ 60 yr)	0.098	0.081-33.058	0.915
Gender	0.895	0.078-41.221	0.389
Primary site	2.135	0.610-18.281	0.078
Tumor differentiation	1.270	0.031-1.672	0.049
UCP2 high expression	4.321	0.035-0.682	0.046

UCP2: Uncoupling protein 2; CI: Confidence interval.

tissue samples (3.92 ± 0.84, n = 15). The expression level of UCP2 protein in colon cancer tissue samples was measured by Western blotting and densitometry, respectively (3.27 ± 0.78, n = 15). A strong linear correlation was found between the T/P ratio of UCP2 mRNA and protein expressions (r = 0.744, P < 0.05), suggesting that increased UCP2 expression in colon cancer tissue is largely determined at the transcriptional level, which is consistent with the reported findings[11].

To characterize the UCP2 protein expression in different human colonic lesions, immunohistochemistry staining was performed for colon tissue sections (including normal colon tissue, non-neoplastic hyperplastic polyp tissue, tubular adenoma tissue, and colon cancer tissue). UCP2 in epithelium of normal colon was not stained. In contrast, strongly positive staining for UCP2 with a diffuse distribution pattern was identified throughout the mucosa from most tubular adenomas and adenocarcinomas. These findings indicate that colonic epithelium is the primary source of increased UCP2

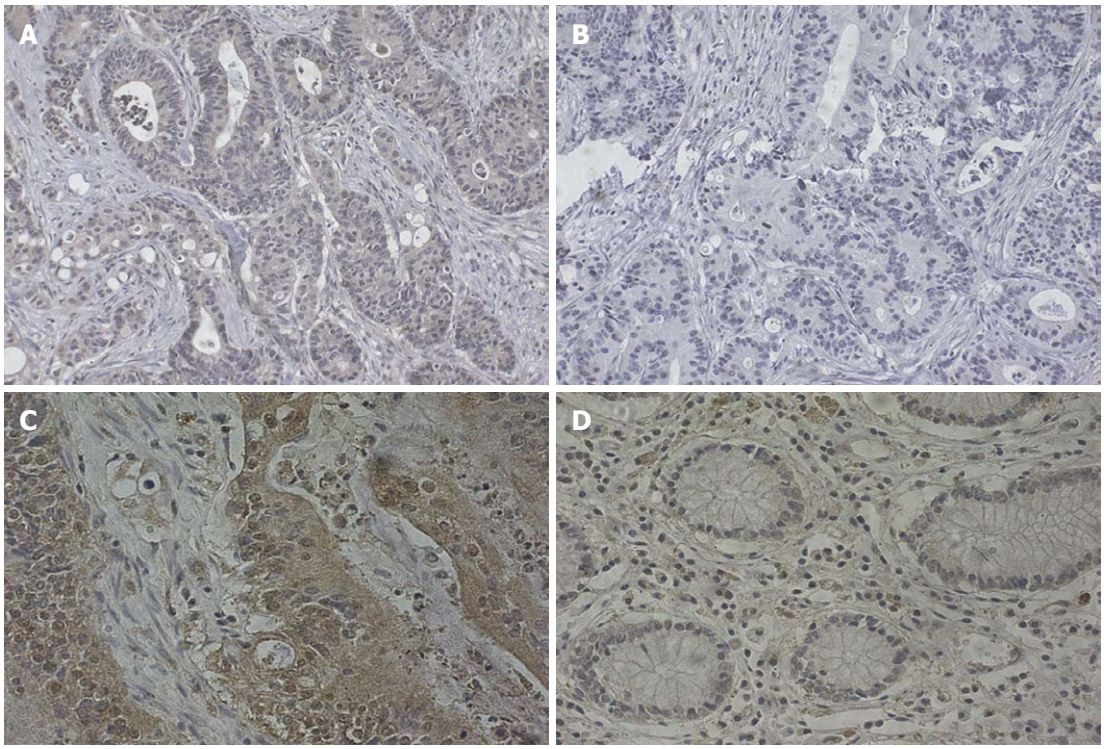

Figure 3 Immunohistological analysis showing uncoupling protein 2 expression in positively stained colon cancer tissue sample (A, C), negative control sample (B), and normal colonic tissue sample (D). Magnification: × 200 (A and B), × 400 (C and D).

expression in colon cancer. The positive UCP2 expression rate was 20% in hyperplastic polyp tissue samples, and over 50% in tubular adenoma samples, and 85.9% in colon cancer tissue samples, respectively, suggesting that UCP2 expression may intensify along the adenoma-carcinoma sequence.

The relation between UCP2 expression and clinical pathological features of colon cancer was analyzed. The expression level of UCP2 was significantly higher in patients with colon cancer at clinical stages Ⅲ and Ⅳ than in those at stage Ⅰ + Ⅱ. However, the UCP2 expression seemed to be irrelevant to cell differentiation status, indicating that further study is needed (Table 5). Furthermore, the relation of colon cancer metastasis with sex and age of patients, tumor location, UCP2 expression and other features was also assessed by univariate analysis, showing that highly expressed UCP2 was significantly correlated to colon cancer metastasis (hazard ratio = 4.321, confidence interval = 0.035-0.682, $P = 0.046$).

Very few studies have reported the potential role of UCP2 in carcinogenesis[12]. Limited literature showed that experimental data are somewhat controversial[13,14]. It has been shown that the UCP2 expression level is moderately higher in human colon cancer cells (LoVo) treated with ionized radiation than in controls not treated with ionized radiation[12,15], and the amount of UCP2 transcripts is greater in apoptosis-sensitive lymphoma cell line after radiation than in apoptosis-resistant cell line[16], suggesting that the decreased mitochondrial membrane potential mediated by UCP2 may activate the cell death pathways[17,18]. Recent reports from multiple laboratories offer a different conclusion[14]. It was reported that drug-resistant tumor cell sub-lines increase the UCP2 expression with a lower mitochondrial membrane potential and a diminished susceptibility to oxidative stress[19-21], indicating that tumor cells may use UCP2 in their metabolic adaptation to avoid ROS-mediated apoptosis.

Over-expression of UCP2 reduces neuronal cell death in transgenic mice and in cell culture exposed to hypoxia and glucose deprivation, coinciding with a decrease in mitochondrial ROS formation[21], and over-expression of UCP2 in cultured cardiomyocytes limits mitochondrial ROS production and suppresses loss of mitochondrial membrane potential elicited by H_2O_2 treatment[22], suggesting that UCP2 over-expression may protect different normal cells against apoptosis, and the cyto-protective role of UCP2 is likely involved in reduction of mitochondrial ROS production.

It has been shown that glycolysis is the preferred energy-producing pathway in rapidly growing cancer cells, while their mitochondrial respiration is diminished[23]. Changes in cancer cell bioenergetics are often associated with more aggressive tumor growth and drug resistance, resulting in worse prognosis[24]. This metabolic switch in cancer cells is to steer away reducing equivalents from the mitochondria to limit ROS generation.

In conclusion, UCP2 is over-expressed in human colon cancer *in vivo*. Increased UCP2 expression in colon cancer is largely determined at the transcriptional level. Highly expressed UCP2 is associated with colon cancer metastasis. Over-expression of UCP2 may be involved in tumor aggressiveness and UCP2 may be a novel target therapy for colon cancer.

COMMENTS

Background

Colon cancer is one of the malignant tumors threatening to human health, and

the mortality of colon cancer patients ranks second among all malignant tumors in developed countries. Its etiology and pathogenesis remains unclear. Adaptive mechanisms in cancer cells include resistance to tumor growth inhibition and evasion of apoptosis, cellular events that are appreciably affected by oxidative stress. Uncoupling protein-2 (UCP2) negatively regulates the production of reactive oxygen species (ROS).

Research frontiers

The etiology and pathogenesis of colon cancer remain unclear. Recent studies showed that mitochondrial dysfunction is involved in the occurrence and development of tumor. UCP2 is a mitochondrial membrane protein, which negatively regulates the production of ROS. The role of UCP2 in development of colon cancer is unclear. In this study, the expression of UCP2 in normal human colon tissue samples and colon cancer tissue samples was detected and the relation between UCP2 expression and clinic-pathological features of colon cancer was analyzed.

Innovations and breakthroughs

Recent studies showed that mitochondrial dysfunction is involved in the occurrence and development of tumor. UCP2 is a member of the inner mitochondrial membrane anion-carrier protein super family and negatively regulates the production of ROS. This is the first study to report the relation between high expression of UCP2 and clinical pathological features of colon cancer.

Applications

By understanding how the expression of UCP2 in colon cancer and analyzing the relation between UCP2 expression and clinical pathological features of colon cancer, we showed that UCP2 might be a novel target therapy for colon cancer.

Terminology

UCP2 is a member of the inner mitochondrial membrane anion-carrier protein super family and negatively regulates the production of ROS. Cancer cells acquire drug resistance as a result of selection pressure dictated by unfavorable microenvironments. This survival process is facilitated through efficient control of oxidative stress originating from mitochondria that typically initiates programmed cell death. This critical adaptive response in cancer cells is linked to UCP2, a mitochondrial suppressor of reactive oxygen species.

Peer review

The authors detected the expression of UCP2 and the relation between UCP2 expression and clinical pathological features of colon cancer. The authors showed that UCP2 expression was increased in colon cancer, and UCP2 might be involved in colon cancer metastasis.

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World Journal of Gastroenterology

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
San Francisco, CA, United States
The Medical Management of HIV/AIDS

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Instructions to authors

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Key words

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Text

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Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use

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Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

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REFERENCES

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Format

Journals

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

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

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 Pixudiarrhoea. *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).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
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Contents

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Contents

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EDITORIAL

A candidate targeting molecule of insulin-like growth factor-Ⅰ receptor for gastrointestinal cancers

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Abstract

Advances in molecular research in cancer have brought new therapeutic strategies into clinical usage. One new group of targets is tyrosine kinase receptors, which can be treated by several strategies, including small molecule tyrosine kinase inhibitors (TKIs) and monoclonal antibodies (mAbs). Aberrant activation of growth factors/receptors and their signal pathways are required for malignant transformation and progression in gastrointestinal (GI) carcinomas. The concept of targeting specific carcinogenic receptors has been validated by successful clinical application of many new drugs. Type I insulin-like growth factor (IGF) receptor (IGF-IR) signaling potently stimulates tumor progression and cellular differentiation, and is a promising new molecular target in human malignancies. In this review, we focus on this promising therapeutic target, IGF-IR. The IGF/IGF-IR axis is an important modifier of tumor cell proliferation, survival, growth, and treatment sensitivity in many malignant diseases, including human GI cancers. Preclinical studies demonstrated that downregulation of IGF-IR signals reversed the neoplastic phenotype and sensitized cells to anticancer treatments. These results were mainly obtained through our strategy of adenoviruses expressing dominant negative IGF-IR (IGF-IR/dn) against gastrointestinal cancers, including esophagus, stomach, colon, and pancreas. We also summarize a variety of strategies to interrupt the IGFs/IGF-IR axis and their preclinical experiences. Several mAbs and TKIs targeting IGF-IR have entered clinical trials, and early results have suggested that these agents have generally acceptable safety profiles as single agents. We summarize the advantages and disadvantages of each strategy and discuss the merits/demerits of dual targeting of IGF-IR and other growth factor receptors, including Her2 and the insulin receptor, as well as other alternatives and possible drug combinations. Thus, IGF-IR might be a candidate for a molecular therapeutic target in human GI carcinomas.

Key words: Dominant negative; Gastrointestinal cancer; Insulin like growth factor-I receptor; Monoclonal antibody; Tyrosine kinase inhibitor

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INTRODUCTION

Signals from a variety of growth factors and their receptors are required for tumorigenesis, cancer development, and maintenance of the malignant phenotype[1]. Those signals alter regulation of the cell cycle, induction of apoptosis, and interactions of tumor cells with their environment, which affect the continuous growth potential of gastrointestinal (GI) cancer cells[1].

Recently, advances in molecular cancer research have brought new therapeutic arms from the bench into clinical usage. One group of new targets is the receptor tyrosine kinases (RTKs), including epidermal growth factor receptor (EGFR, erbB1), Her2/neu (c-erbB2), c-Kit (stem cell factor receptor), and vascular endothelial growth factor receptor (VEGFR). RTKs can be blocked by small molecule tyrosine kinase inhibitors (TKIs), for example gefitinib[2] and imatinib[3], targeting EGFR and c-kit, respectively. Multikinase inhibitors are also available for several tumors, including sorafenib (targeting Raf, VEGFR, PDGFR, c-kit, Flt-3, and RET)[4] and sunitinib (targeting for Flt-3, c-kit, VEGFR, and PDGFR)[5]. RTK signals can be inhibited by human or humanized monoclonal antibodies (mAb), e.g. trastuzumab[6] and cetuximab[7], targeting Her2 and EGFR, respectively. Bevacizumab is a mAb against VEGF-A, which is a ligand for VEGFRs, and is also in clinical use for patients with colorectal cancer[8]. Insulin-like growth factor (IGF) receptor-I (IGF-IR) could be the next molecular target in RTKs of human neoplasms[9].

INSULIN-LIKE GROWTH FACTOR/IGF-I RECEPTOR AXIS

IGF-IR is synthesized as a single precursor peptide of 1367 amino acid residues, which is then cleaved at residue 706, into the α-chain (containing the extracellular domain) and the β-chain (having the transmembrane and tyrosine kinase domains) (Figure 1)[10]. IGF-IR is transported to the membrane fully assembled in the dimeric form with two α-chains and two β-subunits. IGF-I and IGF-II are the ligands of IGF-IR and are produced by the liver and by many extrahepatic sites, including tumor cells and stromal fibroblasts. After the ligands bind to IGF-IR, which is autophosphorylated to stimulate tyrosine kinase activity, IGF-IR subsequently phosphorylates intracellular substrates, including insulin receptor substrates-1 to -4 (IRS-1~4) and Shc. These early events activate multiple signaling pathways, including the mitogen-activated protein kinase [MAPK, extracellular signal-regulated kinase (ERK)] and phosphatidylinositide 3-kinase (PI3-K)/Akt-1 (protein kinase B) pathways[11,12]. Those pathways then switch on several cellular functions, including anti-apoptosis, transcription, metabolism, proliferation, growth, and translation.

In normal cells, the IGF/IGF-IR system is controlled by multiple steps (Figure 2)[13]. Growth hormone-releasing hormone (GHRH) stimulates the expression of growth hormone (GH), which is produced in the pituitary gland. GH then stimulates the secretion of IGFs and IGF binding proteins (IGFBPs) from hepatocytes. Activation of IGF-IR is tightly regulated by the amount of the free forms of the ligands, which is controlled by the action of IGFBP and the non-stimulatory receptor type 2 IGF receptor (IGF-IIR, also known as mannose 6-phosphate receptor)[14,15]. IGFBP-1 to -6 circulate and modulate IGF activity by reducing IGFs bioavailability to bind to the IGF-IR. The complex balance between IGFs and IGFBPs is modulated by specific IGFBP proteases, such as matrix metalloproteinase (MMP)[16]. IGFBPs have IGF-independent actions, but their role in cancer is not yet clear. IGF-IIR is also a negative regulator of IGF signaling, and works by as a decoy by binding IGFs.

THE ROLES OF IGF-IR SIGNALS IN HUMAN NEOPLASMS, ESPECIALLY GASTOROINTESTINAL CANCERS

Dysregulation of the IGFs/IGF-IR system has been implicated in the proliferation of numerous tumors[17]. IGF-IR appears to be essential for malignant transformation in certain systems, for example, fetal fibroblasts with a disruption of the IGF-IR gene, while viable, cannot be transformed by the potent oncogene, SV40 T antigen[11,18]. Elevation of serum IGF-I increases the risk of developing several cancers, e.g. colon, prostate, and breast[14,19,20]. In addition, low serum concentration of IGFBP3 increases the risk of cancer[14]. Increased IGF-II expression has been implicated as a biomarker of colorectal cancer risk[21]. Overexpression of IGFs and the receptor, either by gene amplification, loss of imprinting, or overexpression of convertases or transcription factors, have been observed, as well as posttranslational modifications of the IGF-IR by glycosylation. IGF-IR is also important for the maintenance, as well as the initiation, of malignancy[11]. Moreover, reduction of IGF-IR has been shown to induce apoptosis in tumor cells, but produces only growth arrest in untransformed cells[1], implying that receptor blockade might have a greater therapeutic index than strategies targeting fundamental cell mechanisms, such as DNA synthesis or the cell cycle. In support of this, IGF-IR knockout mice are viable (though physically smaller than normal and ultimately die of respiratory failure), indicating that relatively normal tissue development and differentiation can occur in its absence[22].

Exogenous IGFs stimulate the proliferation of colon, gastric, esophageal, hepatocellular, and pancreatic cancer cells, whereas blocking IGF-IR inhibits tumor progression[23-29]. Intestinal fibroblast-derived IGF-II has been shown to stimulate proliferation of intestinal epithelial cells in a paracrine manner[30]. Both IGF-II and IGF-IR expressions are increased in gastrointestinal cancers[23,28,29,31-33]. Soluble IGF-IIR rescues Apc(Min/+) intestinal adenoma progression induced by loss of IGF-II imprinting[15]. Previously, we reported that detection of IGF-II/IGF-IR might be useful for the prediction of recurrence and poor prognosis of ESCC and for selecting patients for IGF-IR targeting therapy[33]. IGF-I has also

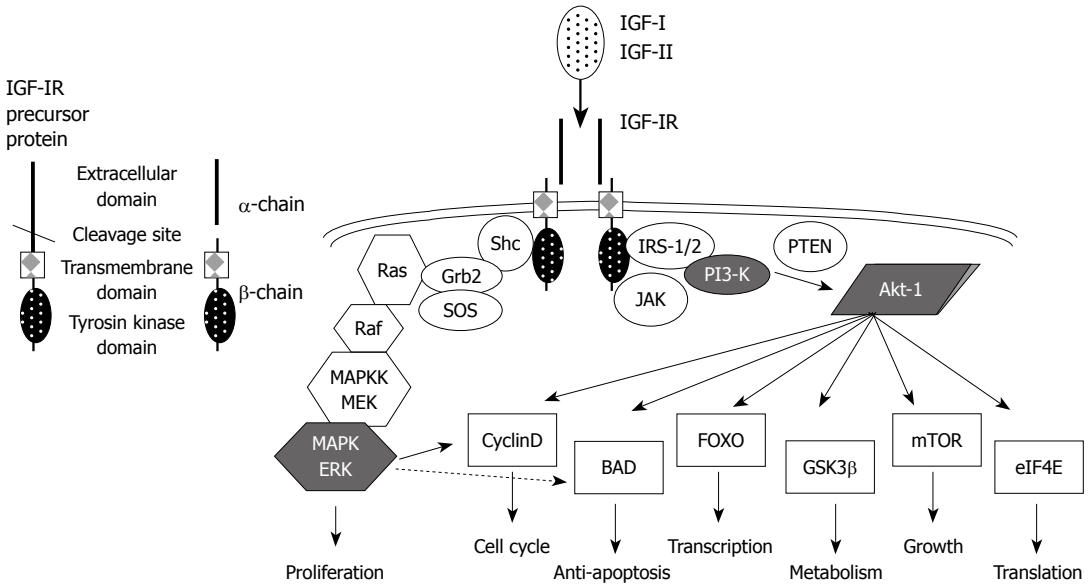

Figure 1 The structure, signal transductions, and effects of the type I insulin-like growth factor receptor system. Type I insulin-like growth factor receptor (IGF-IR) is synthesized as a single precursor peptide and then is cleaved into the α-subunit (extracellular domain) and the β-subunit (transmembrane and tyrosine kinase domains). After binding to the ligands (IGF-I and IGF-II), IGF-IR, which is constructed with two α- and two β-chains, turns on its signal transductions *via* two major pathways, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositide 3-kinase (PI3-K)/Akt, results in survival and mitogenesis. IRS: Insulin receptor substrate; Shc: Src homology and collagen-containing protein; Grb2: Growth factor receptor-bound protein 2; PTEN: Phosphatase and tensin homolog; JAK: Janus kinase; MAPKK: MAPK kinase; MEK: MAPK/ERK kinase; ERK: Extracellular signal-regulated kinase; BAD: Bcl-2-associated death promoter; FOXO: Forkhead box O; GSK3β: Glycogen synthase kinase 3 beta; eIF4E: Eukaryotic translation initiation factor 4E.

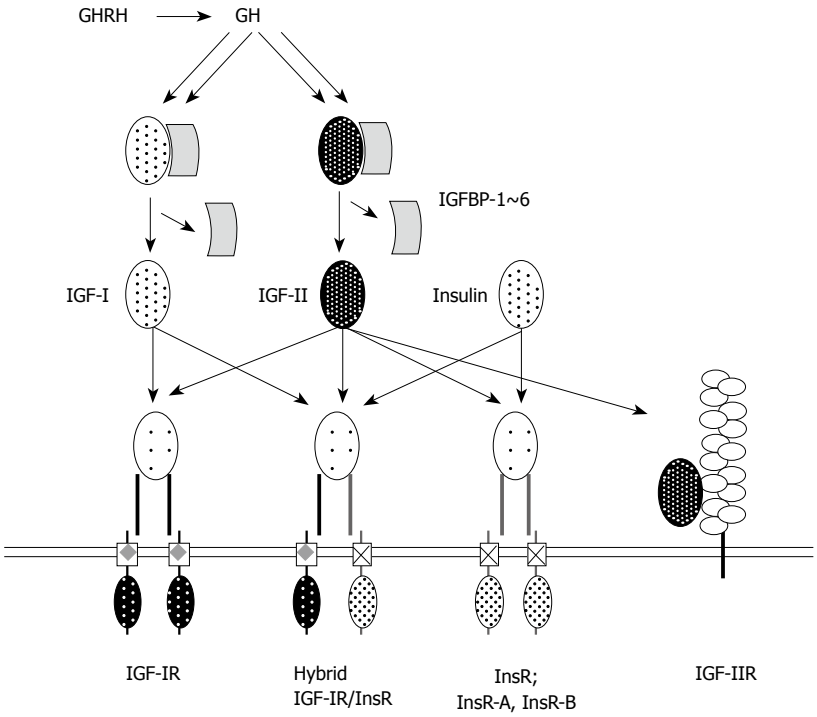

Figure 2 Insulin-like growth factor/type I insulin-like growth factor receptor and insulin/insulin receptor systems. Growth hormone-releasing hormone (GHRH) can stimulate secretion of growth hormone (GH), which upregulates insulin-like growth factors (IGFs) expression. IGF-I and IGF-II, which have about 40% sequence similarity to pro-insulin, predominantly activate type I IGF receptor (IGF-IR), which is a similar structure to insulin receptor (InsR) (59% sequence similarity). IGF-II is able to bind IR and both IGFs can bind hybrid IGF-IR/IR receptors. Ligand supply of both IGFs is regulated by two components. One is IGF binding proteins, which comprise at least six proteins [IGF binding protein (IGFBP)-1~6]. Another is IGF-IIR (lacks tyrosine kinase activity), which internalizes IGF-II for degradation in the pre-lysosomal compartment. Insulin can activate both IR and hybrid IGF-IR/InsR. Two isoforms of InsR exist, the A-isoform (InsR-A) and the B-isoform (InsR-B).

been shown to antagonize the antiproliferative effects of cyclooxygenase-2 inhibitors on pancreatic cancer cells[34]. Thus, overexpressed IGF-IR signals are also important in tumor dissemination through the control of adhesion, migration, and metastasis.

IGF-II, in conjunction with IGF-IR, IGF-I, COX-2, and MMP-7, seems to play a key role in the early stage of colorectal carcinogenesis[35,36]. Matrilysin (MMP-7) can cleave all six IGFBPs and can thus cause increased IGF-mediated IGF-IR phosphorylation[37]. Moreover, matrilysin is also able to generate bioactive IGF-II by degrading the IGF-II/IGFBP-2 complex binding to heparan sulfate proteoglycan in the ECM of HT29[16]. We have previously reported a positive feedback loop between the IGF/IGF-IR axis and matrilysin in the progression and invasiveness of GI cancers[38] (Figure 3).

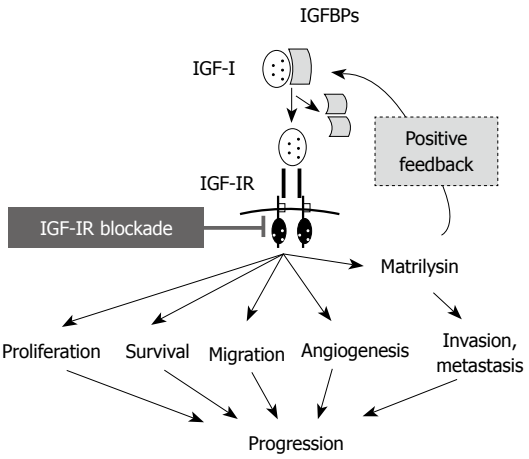

Figure 3 Blockades of type I insulin-like growth factor receptor reduce tumor progression through several interruptions of type I insulin-like growth factor receptor-mediated functions, including type I insulin-like growth factor receptor/matrilysin positive feedback system. IGF-IR: Type I insulin-like growth factor receptor; IGFBPs: IGF binding proteins.

These findings suggest a potential basis for tumor selectivity in therapeutic applications in GI cancers.

INSULIN AND INSULIN RECEPTOR AXIS

The insulin receptor (InsR) is also a key component of the IGF signaling pathway (Figure 2). IGF-IR shares a high degree of sequence similarity to InsR. The ATP binding sites of these two receptors display 100% sequence identity, whereas the entire kinase domains share 84% sequence identity, both with each other and across species[39]. InsR activation leads to cell proliferation in addition to glucose metabolism. In addition to insulin, InsR can also bind IGF-II and initiate mitogenic signaling[40]. In cells that express both receptors, IGF-IR/InsR hybrids form by random association. The hybrid receptors bind both IGF-I and IGF-II at physiological concentrations.

Epidemiological studies linked long standing type 2 diabetes, obesity, and metabolic syndrome with increased risk for developing cancer, including pancreatic and colon cancer[41]. High levels of both insulin and IGF-I are risks for breast cancer in postmenopausal women[12,42]. Phosphorylated IGF-IR/InsR is present in all breast cancer subtypes, and is related to poor survival[43]. InsR and IGF-IR/InsR hybrid receptors might also be involved in cancer biology, as both insulin and IGF-I contribute to the development and progression of adenomatous polyps[44].

Two isoforms of InsR are generated by alternative splicing of exon 11[45]. The A-isoform (InsR-A) is a fetal type and does not contain exon 11, and the B-isoform (InsR-B) is a classic form and contains exon 11[45]. InsR-A can bind IGF-II in addition to insulin and initiates mitogenic signaling[40]. InsR-B is able to bind IGF-I in addition to insulin. Cancers are now known to express InsRs, particularly the fetal variant InsR-A that mediates proliferation and apoptosis protection in response to IGF-II.

THE EFFECTS OF DOMINANT NEGATIVE IGF-IR IN COLORECTAL, GASTRIC, PANCREATIC, AND ESOPHAGEAL CANCER CELLS

Of the many potent strategies targeting the IGF/IGF-IR axis in GI cancer, we will first discuss data generate by our own group[33,38,46-48]. We constructed dominant negative (dn) versions IGF-IR, which can inhibit the function rather than the expression of the naturally expressed receptor[46,49]. We generated two different truncated IGF-IR constructs (950 and 482 amino acid residue IGF-IRs, IGF-IR/950st and IGF-IR/482st, respectively). The former lacks the tyrosine kinase domain and is thought to reside in the membrane of the transduced cells. The latter produces a defective α-chain of IGF-IR and should thus be a secreted form that may affect signal transduction in adjacent cells in addition to the transduced cells. We then constructed adenoviruses expressing two IGF-IR/dns, Ad-IGF-IR/dns (Ad-IGF-IR/482st and Ad-IGF-IR/950st).

In vitro effects and signal transduction of IGF-IR/dn

The Ad-IGF-IR/dns effectively reduced ligand dependent DNA synthesis, an index of mitogenesis, and colony formation, an index of *in vitro* tumorigenicity. IGF-IR/dns induced apoptosis and upregulated stressor (serum starvation, heat, and ethanol)-induced apoptosis.

IGF-IR/482st is a secreted protein and has a bystander effect, which suggest that IGF-IR/482st might enhance antitumor effects.

The IGF-IR/dns reduced ligands-induced phosphorylated Akt-1, but did not influence those of ERKs significantly. IGF-IR/dn can block not only IGF-I but also IGF-II stimulation, broadening the potential activity of IGF-IR/dn as an antitumor therapeutic. Although insulin induced Akt-phosphorylation, IGF-IR/482st did not block this phosphorylation, indicating that Ad-IGF-IR/dn has a high degree of receptor selectivity.

In vivo effects of IGF-IR/dn in GI tumor cells

When the GI cancer cells expressed IGF-IR/dn, the subcutaneous (SC) tumor formation was diminished significantly. Moreover, tumors derived from IGF-IR/dn expressing cells showed limited invasion into the underlying muscle. These results indicate that IGF-IR/dn effectively downregulates *in vivo* tumorigenicity and invasiveness.

Intratumoral (it) injection of Ad-IGF-IR/dn resulted in growth retardation or shrinkage of established GI tumors. The anti-tumor effect of IGF-IR/482st was stronger than that of IGF-IR/950st, undoubtedly due to the bystander effect of IGF-IR/482st. Moreover, IGF-IR/dn suppressed the invasiveness of SC tumors via downregulation of matrilysin expression and increased the number of apoptotic cells in the tumors.

In addition, GI cancer cells form peritoneal tumor

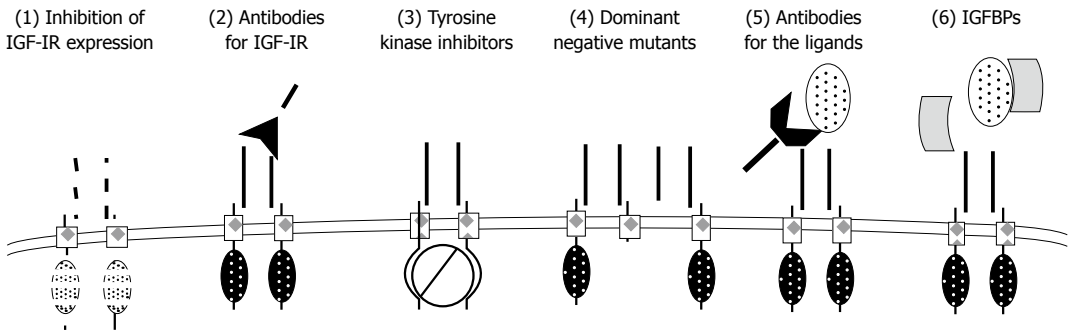

Figure 4 The summary of strategies to inactivate type I insulin-like growth factor receptor system. (1) Inhibition of type I insulin-like growth factor receptor (IGF-IR) protein expression by blocking its translation [with antisense oligodeoxynucleotides and short interfering RNA (siRNA)]; (2) IGF-IR function can be blocked with inactivating monoclonal antibodies (mAb); (3) The tyrosine kinase activity of IGF-IR can be abolished with small-molecule inhibitors (TKI); (4) IGF-IR mutants lacking β-subunits can act as dominant-negative (dn) receptors; (5) Ligand availability can be reduced by mAb for IGFs; and (6) IGF binding proteins (IGFBPs) can reduce active forms of IGFs.

nodules after intraperitoneal (ip) transplantation. Tumor bearing mice were treated by administration (ip) of Ad-IGF-IR/482st. IGF-IR/dn reduced the number of masses and resulted in a significant prolongation of survival in these mice, indicating that IGF-IR/dn can prevent and treat peritoneal cancer dissemination.

Combination effects with chemotherapy or radiotherapy

IGF-IR/dn enhanced chemotherapy (5FU and cisplatin)-induced apoptosis in GI cancer cells. The effects of the combinations were greater than addition of the effects of each monotherapy. IGF-IR/dn also upregulated radiation induced apoptosis. The combination of IGF-IR/482st (it) and 5FU (ip) for established SC tumors on mice was more effective than each single compound, and one-third of masses on the mice treated with this combo were cured; neither monotherapy cured any masses. This indicates that IGF-IR/dn has the potential to enhance the effectiveness of standard cancer therapies. Primary resistance to cytotoxic drugs is a serious problem in GI carcinomas and this approach has the potential to overcome this lack of responsiveness.

METHODS FOR THE BLOCKADE OF IGF/IGF-IR AXIS

Figure 4 shows six methods of disrupting IGF/IGF-IR signaling in cancer: (1) Blocking IGF-IR translation [with antisense (AS) oligodeoxynucleotides, AS RNA constructs, and RNA interference (RNAi)] or transcription (with triple helices) can bring about reduction or elimination of IGF-IR protein expression; (2) Binding of mAbs to IGF-IR can abolish its function; (3) Small-molecule TKIs can reduce the activity of IGF-IR; (4) Defective IGF-IRs, either mutated or lacking the tyrosine kinase domain, can act as dn receptors; (5) mAbs for the ligands can reduce their binding to the receptor; (6) Excess IGF binding proteins or inhibition of ligand expression (at the transcriptional or post-transcriptional level) can reduce the active ligands. There are several other ways to inactivate IGF-IR signals[50]; (7) IGF mimetic peptides can compete with the natural ligands; (8) Expression of a myristoylated IGF-IR C-terminus, which is a domain with intrinsic pro-apoptotic activity, can downregulate signals; (9) GHRH antagonists could diminish IGF-I levels[51]; and (10) Adnectins™ (Bristol-Myers Squibb), a novel class of targeted biologics, are proteins designed to either block or stimulate therapeutic targets of interest[52]. Recently, an optimized Adnectins specific that specifically blocks IGF-IR has been developed.

Agents useful for blocking IGF/IGF-IR signaling in cancer are listed in Table 1. Two of the ways to inhibit IGF-IR expression are RNAi technology and the AS technique[53,54]. We constructed a recombinant adenovirus expressing an AS to IGF-IR that decreases receptor numbers and inhibits soft agar colony forming efficiency, and treatment with this virus can significantly prolong the survival of nude mice bearing human lung tumor xenografts[55]. ATL-1101 (Antisense therapeutics) is an AS oligodeoxynucleotide and was developed for the treatment of psoriasis (stopped after a phase I study)[56]. We have also reported that adenoviral vectors expressing this short-hairpin RNA from IGF-IR induced effective IGF-IR silencing in lung and five GI cancers, as manifested by effective blocking of the downstream pathway of IGF-IR and by antitumor effects[57,58]. Although an adenoviral vector has several advantages, certain side effects have been reported for gene therapy using adenovirus vectors. Thus, there are some unsolved hurdles in practical application.

Many mAbs for IGF-IR had been developed over the years. Although αIR3[59] is a famous mAb for IGF-IR and inhibited cancer cell growth *in vitro*; however, it did not inhibit xenograft growth of breast cancer cell, MCF-7[60]. Thus, none of the first generation mAbs had the precise characteristics for clinical use. Recently, great advances have been in the cloning and production of mAbs by several pharmaceutical companies, e.g. figitumumab (CP-751,871)[61] by Pfizer, SCH 717454[62] by Schering-Plough, IMC-A12[63] by imClone systems, R1507[64] by Roche, AMG 479[65] by AMG, BIIB022[13] by Biogen Idec, MK-0646[66] by Merck, and AVE1642[67] by Sanofi-Aventis. The first six are whole human type mAbs and the latter two are humanized mAbs. These mAbs may have the qualities necessary for clinical usage and currently under phase study. Current IGF-IR targeting mAbs seem to

Table 1 Type I insulin-like growth factor receptor targeting agents

Class	Name	Company	Other targets than IGF-IR	Clinical study	Target organs of GI	Target organs other than GI
Inhibition of IGF-IR expression	Antisense oligonucleotide Antisense RNA siRNA					
Antibodies for IGF-IR	Figitumumab (CP-751,871)[1]	Pfizer		Phase III	Colon	Lung, head and neck, breast, prostate, sarcoma, advanced solid tumor
	SCH 717454 (19D12)[1]	Schering-Plough		Phase II	Colon	sarcoma, advanced solid tumor
	IMC-A12[1]	ImClone Systems		Phase II	Colon, HCC, pancreas, islet cell cancer	Lung, head and neck, breast, prostate, kidney, thymic, adrenocortical, sarcoma, advanced solid tumor, CMPD, leukemia, lymphoma
	R1507 (RG1507)[1]	Roche		Phase II		Lung, breast, sarcoma, advanced solid tumor
	AMG 479[1]	Amgen		Phase II	Colon, pancreas, carcinoid, neuroendocrine cell	Lung, ovarian, prostate, sarcoma, advanced solid tumor
	BIIB022[1]	Biogen Idec		Phase I	Liver	Lung, solid tumor
	MK-0646 (h7C10)[2]	Merck		Phase II	Colon, pancreas, neuroendocrine cell	Lung, breast, myeloma, advanced solid tumor
	AVE 1642[2]	Sanofi-Aventis		Phase II	Liver	Breast
Tyrosine kinase inhibitors	NVP-AEW541[3]	Novartis				
	NVP-ADW742[3]	Novartis				
	NVP-TAE226[3]	Novartis	FAK			
	BMS-536924[3]	Bristol Myers Squibb				
	BMS-554417[3]	Bristol Myers Squibb	IR			
	BMS-754807[3]	Bristol Myers Squibb	IR	Phase I/II	Colon	Breast, head and neck, advanced solid tumor
	EGCG (tea polyphenol)[3]			Phase II	Esophagus	Lung, breast, prostate, bladder, leukemia
	OSI-906 (PQIP)[3]	OSI pharma		Phase III	Colon, liver	Adrenocortical, ovarian, breast, advanced solid tumor
	A-928605[3]	Abbott				
	XL-228	Exelixis	Abl, SFK, Src, Aurora kinase A	Phase I		CML, lymphoma, cancer
	BVP-51004 (cyclolignan PPP)[4]	Biovitrum				
	INSM-18[4] [Nordihydroguaiaretic acid (NDGA)]	INSMED	Her2	Phase II		Prostate, brain, advanced solid tumor, leukemia, MDS, lymphoma
Dominant negative mutants	IGF-IR/482st IGF-IR/486STOP IGF-IR/950st IGF-IR/952STOP					
Antibodies for the ligands	KM1468	Kyowa Hakko				
	KM3168	Kyowa Hakko				
	KM3002	Kyowa Hakko				
IGFBPs	Recombinant human IGFBP3 protein					

[1]Fully human antibody; [2]Humanized antibody; [3]Adenosine triphosphate (ATP) antagonists; [4]Non-ATP antagonists. IGF-IR: Type I insulin-like growth factor receptor; GI: Gastrointestinal; HCC: Hepatocellular carcinoma; CMPD: Chronic myeloproliferative disorder; FAK: Focal adhesion kinase; CML: Chronic myeloid leukaemia; MDS: Myelodysplastic syndromes; IGFBP: IGF binding protein.

share a common mechanism of drug action, namely to blocking ligand binding, decreasing cell surface receptor expression through receptor internalization, and blocking intracellular signaling, particularly through the PI3K/Akt pathway[63,68]. Most mAb are IgG1 class, humanized or fully human, to reduce immunogenicity. IgG1 and IgG3 classes can mediate antibody-dependent cellular cytotoxicity[63,68], which might strengthen anticancer activity and lymphocytic toxicity through recruitment of immune effector cells to antibody-antigen complexes. However, IGF-IR-mAb-directed cellular cytotoxicity could also enhance toxicity to normal IGF-IR-bearing tissues. As

CP-751,871 is an IgG2 subtype, which are usually poor activators of cellular immune response, and BIIB022 is a nonglycosylated IgG4 antibody, ongoing clinical studies should clarify whether these agents have significantly different properties from IgG1 class.

Small molecular TKIs for IGF-IR are synthesized by several companies. Novartis pharma produced three agents, NVP-ADW742[69], NVP-AEW541[70], and NVP-TAE226[71], which has dual targets on IGF-IR and focal adhesion kinase (FAK). Bristol-Myers-Squibb constructed three materials, a specific inhibitor of IGF-IR, BMS-536924[72], and dual inhibitors for InsR and IGF-IR, BMS-554417[73] and BMS-754807[74]. OSI-906[75] is made by OSI pharma and A-928605[76] by Abbott. Tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) is also identified as a TKI for IGF-IR[77]. These nine medicines inhibit IGF-IR kinase activity by an ATP-competitive mechanism. In contrast, there are two compounds that are IGF-IR TKI non-ATP antagonists, cyclolignan picropodophyllin (PPP)[78] and INSM-18[13]. The latter is a dual TKI for IGF-IR and Her2. XL-228 is a multi-target TKI for IGF-IR, Bcl-Abl, SFK, Src, and Aurora kinase A[13]. At least five TKIs are currently in clinical studies.

We used two dn inhibitors of IGF-IR, as detailed above. Several groups have used IGF-IR/486STOP[79,80] and IGF-IR/952STOP[81,82], the former resembles our IGF-IR/482st and the latter is similar to our IGF-IR/950st.

mAbs for IGF-I and IGF-II, KM1468[83,84], KM3168[85], and KM3002, are made by Kyowa Hakko. KM1468 neutralized both ligands and inhibited bone metastasis in an animal model.

The last approach is a recombinant human IGFBP-3 protein, which is available for intravenous injection[86] and is beginning clinical testing.

TOXICITY AND COMBINATION OF IGF-IR TARGETING STRATEGIES

The two major potential toxicities of IGF-IR blockade are based on the IGF-IR expression in normal tissues and homology between IGF-IR and InsR. Long-term IGF-IR blockade might cause growth retardation during childhood and might influence the function of IGF-dependent tissues, including the myocardium and brain at any age[87,88]. IGF-IR-inhibitory drugs are predicted to influence glucose tolerance. TKIs might directly inhibit the InsR kinase in some degree, because of binding to a well-conserved ATP binding pocket. In fact, some TKIs can inhibit both receptors, e.g. NVP-TAE226, BMS-554417, and INSM-18. Several anti-IGF-IR mAbs, such as scFv-Fc and EM164, might induce downregulation of InsR *via* endocytosis of hybrid receptors or InsR, which was observed in cancer cells expressing both receptors, but not in cells expressing InsR only[89]. This suggests that anti-IGF-IR mAbs will not inhibit InsR function in insulin-responsible tissues, e.g. hepatocytes, which do not express IGF-IR. In addition, both IGF-IR mAbs and TKIs might result in loss of the hypoglycemic effects of IGF-I, and blockade of pituitary IGF-IRs might result in a compensatory increase in serum concentration of GH, which could contribute to insulin resistance[90].

Although IGF-IR mAbs are exquisitely specific inhibitors of receptor function (by inducing rapid internalization and down-regulation of the receptor), TKIs suffer from a lack of selectivity. TKIs, in general, do not lead to internalization or downregulation of IGF-IR, and will probably represent a broad spectrum of specificity against IGF-IR and InsR and a unique profile of toxicity. Possible toxicity of the central nervous system deserves particular attention during treatment with TKIs, because other molecules in this class have been shown to infiltrate the blood-brain barrier in central nervous system tumors[91]. Nevertheless, IGF-IR TKIs offer several advantages, such as oral administration and of the ability to control the duration of drug exposure, in contrast to long-acting mAbs.

Recently, it has been revealed that the insulin/InsR axis has certain roles in carcinogenicity and tumor development. Chronic hyperinsulinaemia might be a cause of colon and pancreas cancers[92]. IGFs have some potential for binding to and activating InsR. In addition, hybrid receptors of IGF-IR and InsR exist on malignant cells. Thus, blockade of InsR is another matter of concern to eliminate cancer cells. Thus, dual targeting TKIs for IGF-IR and InsR have merits for terminating tumor cells; however, they would, again, have adverse effects of glucose homeostasis.

According to several clinical studies, it has been reported that the adverse effects of IGF-IR mAb are hyperglycemia, mild skin toxicities (rash, flushing, pruritus, and acne), and fatigue as common toxicities of these antibodies[64,93,94]. Other observed toxicities, such as CD4+ lymphocytopenea, thrombocytopenia, and transaminitis, do not seem to be related to the mechanism of their specific action. An IGF-IR mAb caused hyperglycemia in around 20% of patients, but was tolerable, mild to moderate (grades 1 and 2), reversible, and manageable with oral hypoglycemic drugs. Patients with previous glucose intolerance or with steroids usage were at risk of hyperglycemia.

IGF-IR is a mediator of resistance to therapy. IGF-IR activation is known to protect tumor cells against apoptosis induced by cytotoxic drugs, and might also influence the repair of DNA damage[95,96]. There is considerable preclinical data to support the view that IGF-IR inhibition can enhance sensitivity to chemotherapy and radiotherapy. In addition, blockade of IGF-IR might have combination effects with other molecular targeting therapies, especially for RTKs.

Recently, a new role for IGF-IR has been proposed in that its signals might be an escape pathway in cancer cells for drug resistance. Many patients who achieve an initial response to trastuzumab acquire resistance within one year of treatment initiation. Two mechanisms for this trastuzumab resistance have been reported; one is overexpression of IGF-IR[97] and the other is the formation of a IGF-IR/Her2 heterodimer[98]. In addition, IGF-induced PI3-K/Akt activation mediates resistance to EGFR blockade in glioblastomas[99].

Thus, there is a hypothesis for horizontal blockade of two different growth factor receptors, such as Her/EGFR and IGF-IR. Several groups have tried these dual targeting therapies. Recently, a candidate combination treatment with an IGF-IR TKI, BMS-536924, and EGFR/Her2 inhibitors was reported[100].

On the other hand, nonselective inhibitors might have a different profile and alternative benefits. Some TKIs inhibit other kinases, such as Src (XL-228) or Her2 (INSM-18), and these multi-kinase inhibitors can expand the activity of the agent. It could also add toxicity mediated by target and off-target effects, complicating the combination with other agents.

Treatment with CP-751,871 decreased both total circulating tumor cell count and IGF-IR-positive circulating tumor cell count, suggesting that circulating tumor cells could be used as a biomarker of drug effect[93]. High concentrations of serum free IGF-I might be a marker of high responder of patients with non small cell lung carcinoma treated with figitumumab.

Apart from mAbs and TKIs, there are individual approaches using short interfering RNA (siRNA), peptides, proteins, or antisense oligonucleotides to antagonize IGF-IR. As mentioned above, several group, including ours, have revealed that both dn and siRNA for IGF-IR show powerful anti-tumor effects. However, the delivery systems of these approaches represent a significant hurdle for clinical use. Given a suitable delivery tool for humans, we would want to start using both dn and siRNA for IGF-IR in the patients with GI cancer.

CONCLUSION

The IGF/IGF-IR axis plays pivotal roles in the carcinogenicity and progression of GI cancers. We have presented the efficacy of IGF-IR targeting strategies using our data of IGF-IR/dn against GI cancers. Blockade of IGF-IR suppresses carcinogenicity, and upregulates apoptosis-induction and the effects of chemotherapy, both *in vitro* and *in vivo*. We summarized several approaches to blocking IGF-IR signals and discussed the merits and demerits of each strategy. In addition to combination with classical chemotherapy, several attempts at dual targeting for IGF-IR and other growth factor receptors have been made. Many drugs blocking IGF-IR function are now entering clinical trials. Thus, IGF-IR might be a candidate therapeutic molecular target in gastrointestinal malignancies.

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EDITORIAL

Pathobiology of the neutrophil-intestinal epithelial cell interaction: Role in carcinogenesis

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Abstract

The role of chronic inflammation, acting as an independent factor, on the onset of gastrointestinal carcinogenesis is now well accepted. However, even if there is an increase in the number of elements directly involving polymorphonuclear leukocytes (PMNL), as a major actor in digestive carcinogenesis, the different cellular and molecular events occurring in this process are still not completely understood. The transepithelial migration of PMNL, which is the ultimate step of the afflux of PMNL into the digestive mucosa, is a complex phenomenon involving sequential interaction of molecules expressed both on PMNL and on digestive epithelial cells. Chronic inflammatory areas rich in PMNL [so-called (chronic active inflammation)] and iterative transepithelial migration of PMNL certainly evoke intracellular signals, which lead toward progressive transformation of epithelia. Among these different signals, the mutagenic effect of reactive oxygen species and nitrates, the activation of the nuclear factor-κB pathway, and the modulation of expression of certain microRNA are key actors. Following the initiation of carcinogenesis, PMNL are involved in the progression and invasion of digestive carcinomas, with which they interact. It is noteworthy that different subpopulations of PMNL, which can have some opposite effects on tumor growth, in association with different levels of transforming growth factor-β and with the number of CD8 positive T lymphocytes, could be present during the development of digestive carcinoma. Other factors that involve PMNL, such as massive elastase release, and the production of angiogenic factors, can participate in the progression of neoplastic cells through tissues. PMNL may play a major role in the onset of metastases, since they allow the tumor cells to cross the endothelial barrier and to migrate into the blood stream. Finally, PMNL play a role, alone or in association with other cell parameters, in the initiation, promotion, progression and dissemination of digestive carcinomas. This review focuses on the main currently accepted cellular and molecular mechanisms that involve PMNL as key actors in digestive carcinogenesis.

Key words: Neutrophils; Intestinal epithelial cells; Carcinogenesis; Cytokines; Nuclear factor-κB pathway; MicroRNA; Reactive oxygen species

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INTRODUCTION

The link between a chronic active inflammatory process (i.e. chronic inflammation rich in neutrophils) and the onset of carcinoma, in association or not with another factor such as a pathogen, is now convincingly demon-

strated with epidemiological, experimental, and molecular data obtained for different tissues[1-10]. In particular, this relationship is well-established at the gastric and intestinal mucosal level[11-18]. Different factors are involved in digestive carcinogenesis, but the association of these factors and their importance in cancer onset are certainly variable from one disease to another and among individuals. Thus, predisposing genetic factors, infectious factors and inflammatory factors can be involved in digestive carcinogenesis[19]. Inappropriate innate immunity induces cellular infiltration of the digestive mucosa composed of polymorphonuclear leukocytes (PMNL), dendritic cells, natural killer cells, and then secondarily, an afflux of adaptive immune cells such as T lymphocytes. The intensity of this polymorphous cellular infiltrate varies according to the period of the active phases of the digestive disease[20]. In this regard, inflammatory infiltration can be present at variable time periods and at a variable frequency. Among the different populations of cells which migrate into the digestive mucosa, the PMNL play a central role in the pathophysiology of inflammatory digestive diseases[21]. Thus, previous epidemiological and histological studies have convincingly demonstrated a direct link between the clinical symptoms (pain and diarrhea) and the presence of PMNL in the digestive mucosa. More particularly, the periods of acute diarrhea certainly correlate with transepithelial migration of PMNL into the digestive lumen. It is noteworthy that during interaction between the intestinal epithelial cells (IEC) and PMNL different intracellular events are triggered, leading to neoplastic transformation of the digestive epithelia. The molecular phases involved in PMNL transepithelial migration are complex, but it is crucial to understand these phases to better comprehend the initial steps in digestive carcinogenesis. The progression from an *in situ* carcinoma to a microinvasive and invasive digestive carcinoma is associated with several molecular events, in particular, cytoskeleton modification, modulation of adherence molecules and metalloprotease production. Among these different events, some directly implicate PMNL. Currently, the pros and cons of the role of PMNL in tumor progression are debatable[22,23]. PMNL produce elastases[24], which favor tumor cell extracellular matrix invasion and release of pro-angiogenic factors, which creates a favorable microenvironment for tumor progression[25-30], but also produce defensins, which have an anti-tumor effect. Recently, a dual function of PMNL, in regard to their action on carcinoma cells, has been proposed[31,32]. Thus, two different populations of PMNL can be present in tumors, a population that favors tumour progression, the tumor-associated neutrophils 1 (TAN1) and a population that decreases tumor progression, the TAN2[31,32]. Accordingly to the proportion of TAN1 and TAN2 in a carcinoma the level of tumor progression can vary. This phenomenon can be present in colonic adenocarcinomas. Finally, previous studies implicate PMNL in the pathophysiology of metastases. This phenomenon can occur in colonic adenocarcinoma dissemination. In particular, PMNL allow transendothelial migration of tumor cells and then their migration into the blood stream.

Previous studies and reviews have focused on the role of the immune system during cancer development[33] but the impact of PMNL in the different phases of the natural history of cancer (Figure 1) has been poorly described to date. In this review, I describe the role of PMNL and the direct events induced by PMNL in the mechanisms of the different steps in digestive carcinogenesis (cancer initiation, progression and dissemination).

THE BIOLOGY OF THE NEUTROPHIL-INTESTINAL EPITHELIAL CELL INTERACTION

After transendothelial migration, following the crossing of the matrix of the lamina propria, which is mainly induced by a gradient of interleukin (IL) 8[34], PMNL adhere to the basal side of the glandular and crypt cell epithelium, and then transmigrate to the digestive lumen. This transepithelial migration is associated with sequential steps and with dynamic and transitory interactions between some surface molecules that are present on cytoplasmic membranes of PMNL and IEC[35,36] (Figure 2). Studies using *in vitro* models, such as the T84 model, have greatly improved our knowledge concerning these different cellular interactions. Thus, PMNL transepithelial migration can be induced by different stresses on epithelial cells, such as bacteria, bacterial products, toxins, or hypoxia[37,38]. Using this T84 model, the different steps of PMNL transepithelial migration and the different mechanisms involved in cell-cell interactions have been described[39-41]. Briefly, PMNL adhere to the basal side of the digestive epithelia through their CD11b/CD18 molecules (for which the ligand on epithelia is still unknown), then they migrate using a paracellular pathway through an homophilic CD47 interaction, which is expressed both on PMNL and IEC[42,43]. A more recent study showed that CD47 regulates neutrophil transmigration through close cross-talk with one toll-like receptor, TLR-2[44]. Other interactions occur at the desmosome and tight junction levels, which involve JAM and SIRPα[45-47]. After crossing the epithelial barrier PMNL interact with ICAM1 at the apical membrane through CD11b/CD18. During this transepithelial migration, the actin cytoskeleton of epithelial cells is reorganized[48]. Activated PMNL release 5'-adenosine monophosphate, which is secondarily cleaved by an epithelial membrane ectonucleotidase into adenosine, and finally produce chloride secretion on the epithelial apical side[49,50]. More recently, other molecular mechanisms have been described to occur during interaction between PMNL and the IEC[44,51]. Serine protease-mediated activation of epithelial protease-activated receptors has been shown to increase permeability. It has been demonstrated that transmigrating PMNL can regulate barrier function through epithelial protease-activated receptor activation[51]. Thus, transepithelial resistance decreased significantly after contact of PMNL with basolateral sur-

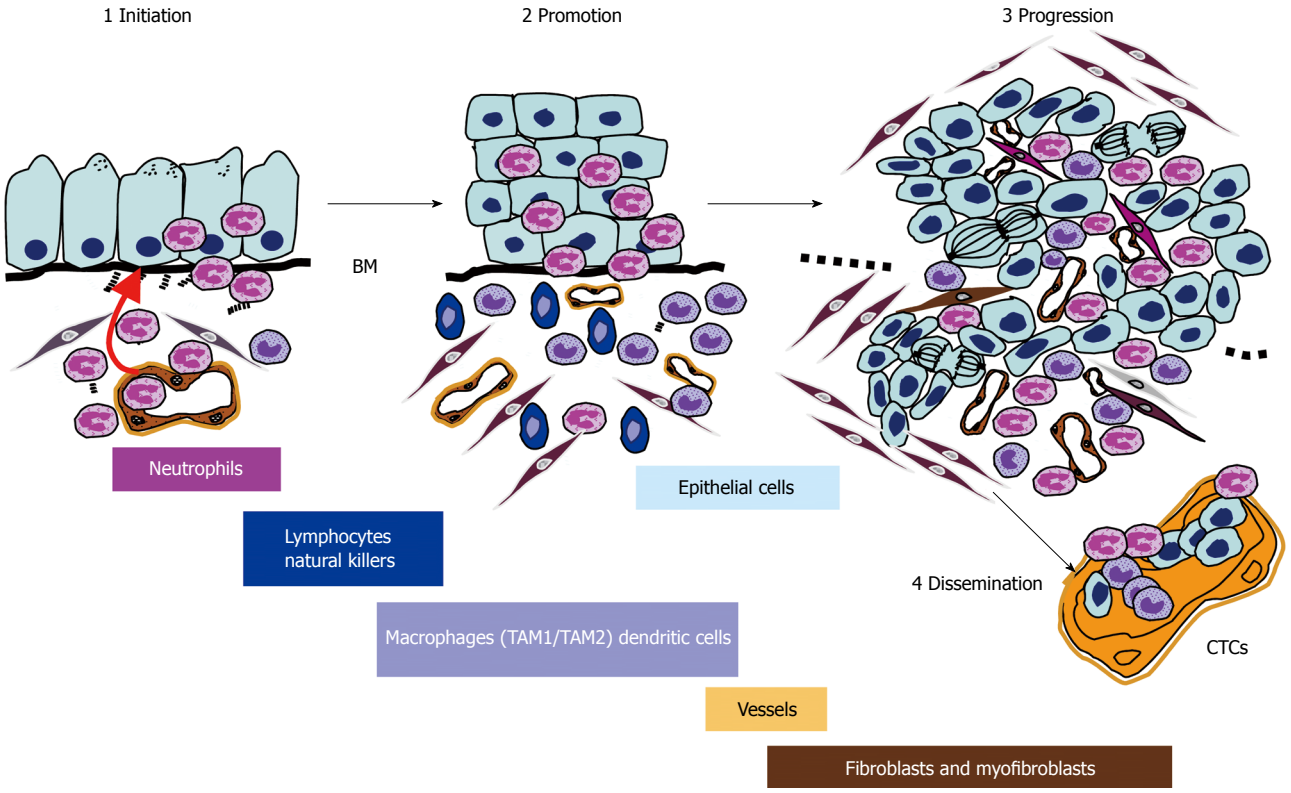

Figure 1 Involvement of a neutrophil-rich microenvironment in the different steps of digestive carcinogenesis including initiation, promotion, progression and dissemination of tumor. BM: Basement membrane; TAM: Tumor-associated macrophages; CTCs: Circulating tumor cells.

faces of T84 monolayers or after incubation with PMNL elastase and proteinase-3[51].

ROLE OF CHRONIC ACTIVE INFLAMMATION IN INITIATION OF DIGESTIVE CARCINOGENESIS

Beside these different events, which are associated with rapid paracellular migration of PMNL, different studies using the T84 model demonstrated the modulation of different molecules expressed on epithelial cells, which may be potentially involved in the initiation of carcinogenesis in direct or indirect pathways, by inducing an amplified inflammatory response rich in PMNL[52,53]. Moreover, paracellular migration of PMNL induced the onset of apoptosis, and, then potentially increases turnover of epithelium regeneration[54]. Thus, there is certainly a tight association between this chronic active inflammation and the onset of digestive carcinoma. An increased level in oxidative stress is present in the mucosa of inflammatory bowel diseases[55-57]. In this regard, an inflammatory microenvironment rich in PMNL can increase the rate of mutation, in addition to enhancing the proliferation of mutated cells[58]. Activated PMNL serve as sources of reactive oxygen species (ROS) and reactive nitrogen intermediates that are capable of inducing DNA damage and genomic instability[59]. Interestingly, release of ROS can occur during epithelium adhesion, but also during transepithelial migration and during post transepithelial migration of PMNL[60]. Alternatively, activated PMNL may use cytokines such as tumor necrosis factor (TNF)-α, which is implicated in carcinogenesis, to stimulate ROS and nitric oxide accumulation in neighboring epithelial cells[61,62]. Moreover, nitric oxide synthase can activate cyclooxygenase-2 in epithelial cells[63]. Different studies focus primarily on the effect of early mediators of inflammation, such as TNF-α, in stimulating tumor cell growth by activating nuclear factor (NF)-κB[64]. Conversely, decreased production of TNF-α in mice can reduce digestive carcinogenesis associated with chronic colitis[65]. However, chronic inflammation involves many other cytokines in the host microenvironment, which may also affect tumor growth in an NF-κB-dependent manner. While most inflammatory cytokines are released from activated macrophages following stimulus-induced transcription, others are secreted from intracellular pools and display later kinetics during the inflammatory response. Furthermore, the fact that NF-κB inhibition does not completely prevent tumor formation in these studies suggests that cytokines could also promote tumorigenesis *via* alternative pathways[66]. Mutations in p53, caused by oxidative damage, were found in both cancer cells and in a non-dysplastic epithelium in cancer associated colitis, suggesting that chronic inflammation causes genomic changes[67]. Finally, ROS can also cause direct oxidative inactivation of mismatch repair enzymes[5].

Other mechanisms have been described, which involve PMNL in the early steps of initiation of carcino-

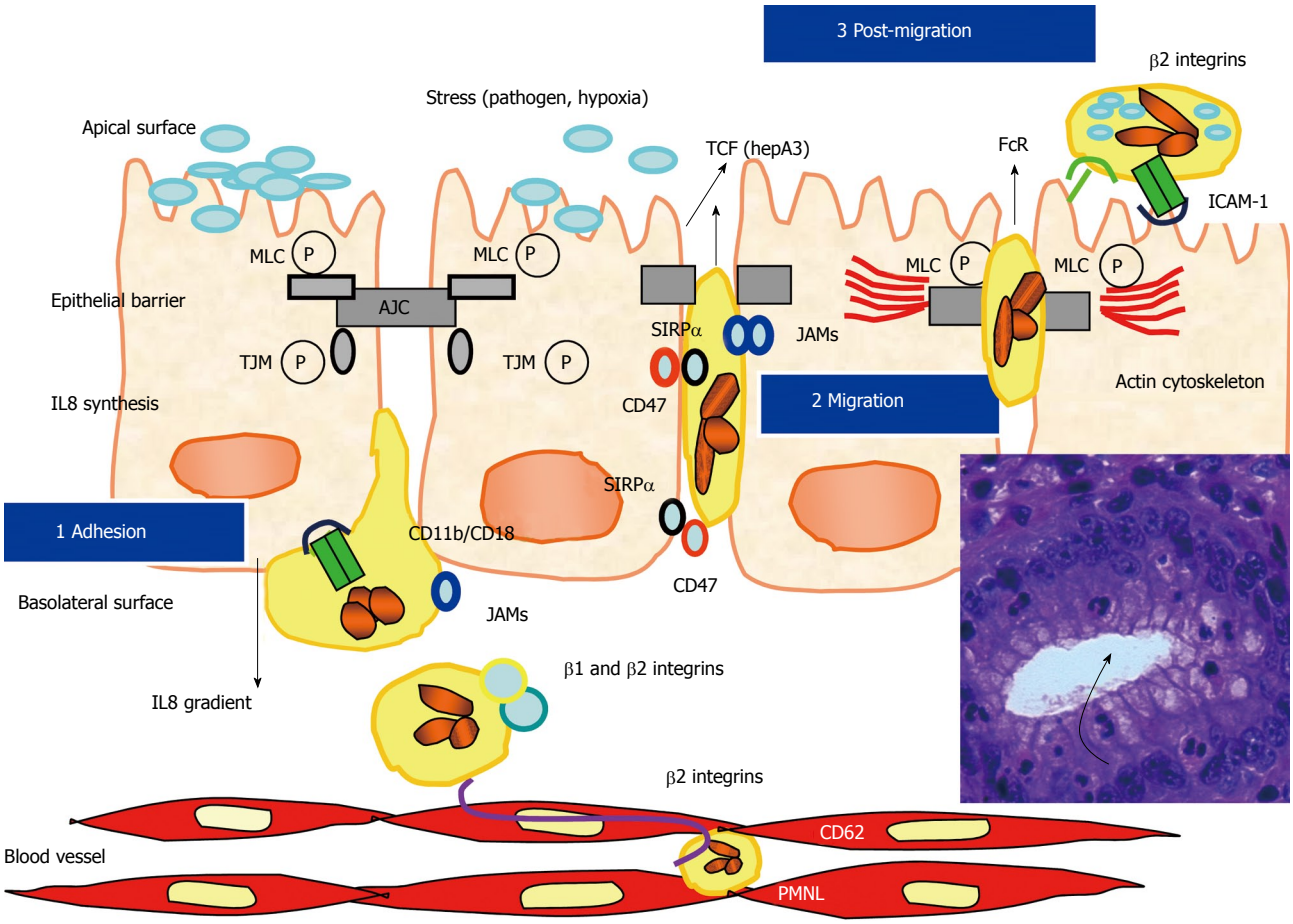

Figure 2 Cross-talk between polymorphonuclear leukocytes and intestinal epithelial cells. Different steps and molecules involvement in polymorphonuclear leukocytes transepithelial migration. The microphotograph shows polymorphonuclear leukocytes (PMNL) in an intestinal epithelium. TJM: Tight junction molecules; AJC: Apical junction complex; MLC: Myosin light chain kinase; JAM: Junctional adhesion molecules; TCF: Transcellular chemotactic factor; SIRP: Signal regulatory protein; IL: Interleukin.

genesis. Using animal models that reproduce digestive carcinogenesis linked to colitis, the molecule vanin 1 has been recently implicated in the onset of carcinoma[68]. Interestingly, it has been described that protein expression of cyclooxygenase-2 and the hypoxia-inducible factor-1 is up-regulated and associated with inflammation in early steps of digestive carcinoma[69]. The role of ROS and nitrates, largely suggested by previous studies, has been highlighted by different recent studies[70-76]. Interestingly, the myeloperoxidase (MPO) released by activated PMNL can inhibit nucleotide excision repair in certain epithelial cell lines[77]. In this regard, mutagenic products of MPO such as 5-chlorouracil and 5-bromouracil are released into inflammatory tissues. Moreover, the role of PMNL in initiation of carcinogenesis is probably more complex[78-80].

MicroRNA have been mainly investigated in oncology. However, microRNA are also implicated in inflammatory mechanisms, and their deregulation during some inflammatory diseases, in particular at the digestive level, could be associated with the molecular events that link chronic inflammation to cancer development[81-87]. The action of PMNL in this process is currently difficult to define, but through ROS release, and/or by the production of different enzymes, PMNL probably participate in deregulation of the RNA network in digestive epithelial cells.

IMPLICATION OF NEUTROPHILS IN PROGRESSION OF DIGESTIVE CARCINOMA

Recent studies have demonstrated that the presence of intratumoral PMNL can be associated with shorter disease specific survival in certain cancer patients[88]. Following the initiation of digestive carcinoma, processes allow the tumor to grow from a single initiated cell into a developed primary adenocarcinoma. In this context, tumor growth depends on increased cell proliferation and reduced cell death, both of which can be stimulated by PMNL-driven mechanisms. This inflammation-induced tumor promotion may occur early or late in tumor development and leads to activation of premalignant lesions that have been dormant for many years. As for tumor-associated macrophages[89-91], PMNL probably promote tumor growth but the putative mechanisms have not yet been determined. However, it has been shown that accelerated intestinal epithelial cell turnover caused by chronic active inflammation and epithelial damage might predispose the mucosa to DNA damage, resulting in an elevated risk of mutation, which is in line with dysplasia and carcinoma development in patients with ulcerative

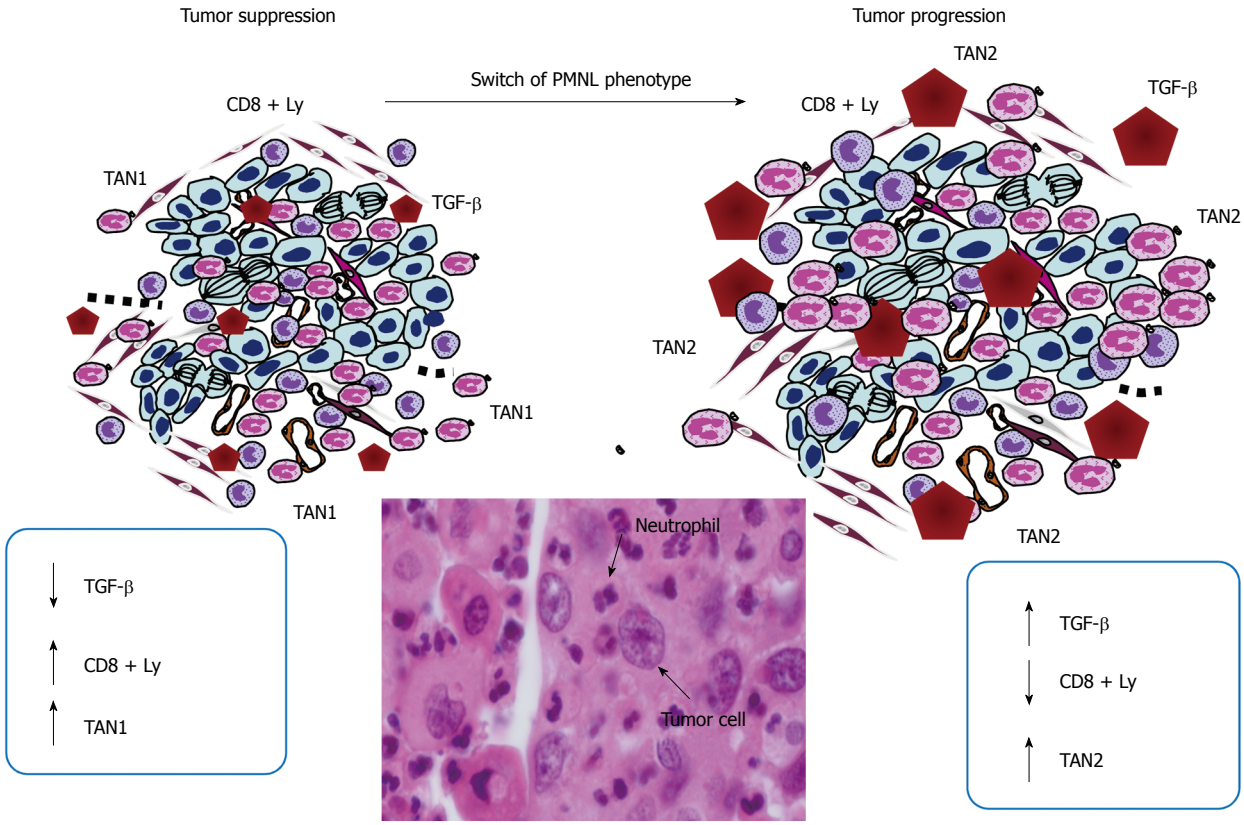

Figure 3 Speculative role of tumor-associated neutrophils in progression of digestive carcinoma. The microphotograph shows neutrophils tightly associated with digestive carcinoma cells. TAN: Tumor-associated neutrophils; TGF: Transforming growth factor.

colitis[92]. In parallel, the repeated inflammatory process could act on COX-2 expression which is down-regulated by the adenomatous polyposis coli (APC) gene and up-regulated by nuclear beta-catenin accumulation, and additionally implicate the Wnt signaling transduction pathway in colon carcinogenesis[93].

Secreted PMNL factors, such as human neutrophil peptides 1-3 (HNP1-3), have been found to be elevated in patients with digestive carcinoma, both in tissues and plasma, and to correlate with Dukes' stages[94]. Other molecules such as neutrophil gelatinase-associated lipocalin or neutrophil elastase are able to suppress or to increase the invasion of carcinoma cells[95-97]. Among the cytokines involved in carcinoma progression, Transforming growth factor (TGF)-β is certainly one of the most studied, to date. It has been reported recently in a mouse model of carcinoma that TGF-β controls maturation of a sub-type of PMNL, the so-called TAN-2. TANs could function in parallel with tumor-associated macrophages (TAMs)[98,99]. Conversely, inhibition of the TGF-β activity leads to the differentiation of PMNL in anti-tumor TAN-1 cells (Figure 3). While TAN-2 inhibit the cytotoxic response of CD8+-T lymphocytes, which infiltrate the intestinal mucosa and thereby allow tumor cells to circumvent immune surveillance, TAN-1 enhance the anti-tumor action of CD8+ T-lymphocytes. TGF-β blockade not only activates CD8+T cells, but also increases the recruitment of hypersegmented neutrophils, their NI polarization (high expression of TNF-α, ICAM-1 and FAS) and their anti-tumor activity. Moreover, N1 neutrophils produce T cell-attracting chemokines including CCL3, CXCL9 and CXCL10. By contrast, TGF-β stimulation polarizes PMNL to the so-called N2 state with increased expression of arginase and chemokines such as CCL2 and CCL5. N1 are cytotoxic for tumors, whereas N2 display pro-tumor properties.

We may speculate that this mechanism is universally found in carcinomas arising in different organs. Finally, it is noteworthy that the prognostic value of a high number of PMNL in different carcinomas correlates with poor outcome in previous studies[100].

In addition to TGF-β, other cytokines produced by PMNL may be involved in carcinoma progression. Thus, TNF-β secreted by PMNL can stimulate a positive loop of inflammation by inducing production of chemokines such as IL8 and Groα by epithelial tumor cells and probably inducing renewed recruitment of PMNL[101]. Moreover, other mechanisms may exist such as carcinoma cell stimulation of PMNL to produce oncostatin M[102].

Although it is not yet established, we can speculate that some miRNA expressed by PMNL, in particular mir-223, may also play a crucial role in modulating progression of digestive tumors. Mir-223 was found to possess a crucial role in regulating neutrophil proliferation and activation[103]. Moreover, the expression of mir-223 may be modulated by some cytokines released by tumor cells and may influence the phenotype of TAN-1 or

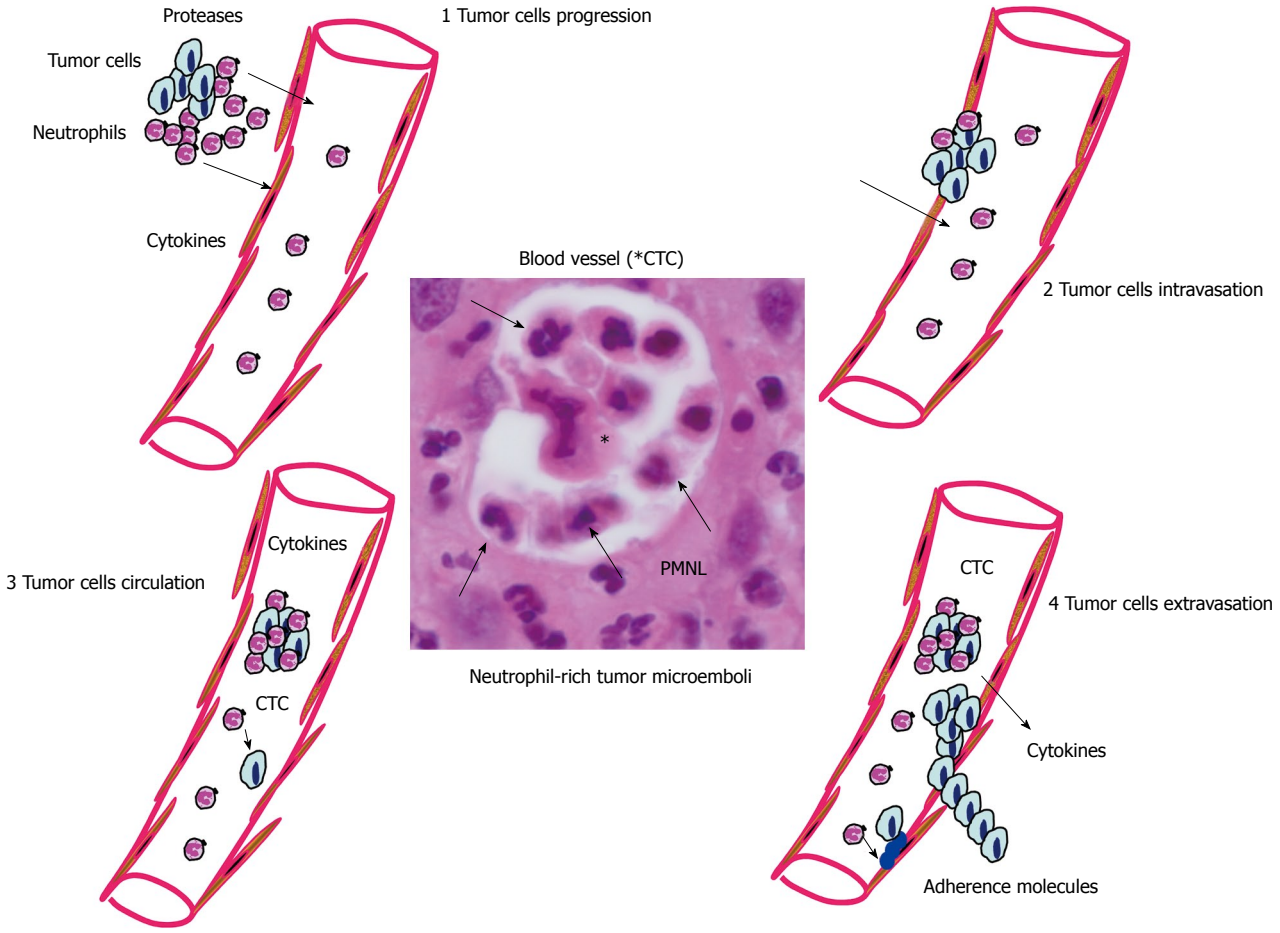

Figure 4 Speculative role of polymorphonuclear leukocytes in digestive carcinoma dissemination. The microphotograph shows circulating tumor cells associated with polymorphonuclear leukocytes (PMNL). CTC: Circulating tumor cells.

TAN-2. In this regard, different molecules have recently been reported as markers and/or promoters of inflammation-associated cancers[104]. Thus, we can speculate that the level of expression of mir-223 in carcinoma might be a marker of tumor progression.

THE NEUTROPHIL AS AN ACTOR OF THE PATHOBIOLOGY OF DIGESTIVE CARCINOMA METASTASIS

Inflammation is a key actor of metastasis onset[105]. In this regard, different studies have demonstrated the role of PMNL in tumor metastasis through different steps[106,107]. PMNL can participate in the transendothelial migration of adenocarcinoma cells, as well as their dissemination into the blood (Figure 4)[108,109]. Cytokines produced by PMNL can increase vascular permeability and upregulation of certain adhesion molecules located on endothelial cells[110]. In addition, PMNL are important sources of proteases that degrade the extracellular matrix and may alter the vascular barrier allowing entry of tumor cells into the blood stream. Interestingly, in a model of invasive colon cancer, CCR1+ myeloid cells, the recruitment of which is driven by the chemokine CCL9 produced by cancer cells, promote invasiveness through secretion of the matrix metalloproteinases MMP2 and MMP9[111]. It has been demonstrated that extracellular ATP can be released by activated PMNL[112]. This release of ATP occurs through a conformational opening of membrane Cx43 hemichannels in response to PMNL activation[113]. Moreover, the extracellular ATP released by activated PMNL may act both on epithelial cells, through activation of some purinergic receptors expressed by epithelial cells[53], and on endothelial cells[112]. More specifically, ATP released by activated PMNL is auto-hydrolyzed to AMP through CD39 on the surface of PMNL. CD39 may function as an immunomodulatory control point, requiring a close and special relationship with CD73-positive cells, such as endothelial cells. In addition to regulating the endothelial barrier function, a role for PMNL-dependent ATP release in directed movement of PMNL has been reported[114]. ROS released by activated PMNL can generate mitochondrial DNA mutations that regulate tumor cell metastasis[115].

Once metastatic cells enter the circulation, they need to survive in suspension and resist detachment-induced cell death or anoikis. The survival of circulating cancer cells is affected by inflammatory mediators released by immune cells in response to cancer-derived stimuli[116]. In the same way, the presence of a variety of cytokines

released by activated PMNL present in the tumor microenvironment, including TNF-α, can promote the survival of circulating metastatic seeds[117]. PMNL can also favor the circulation in the blood of tumor cells, in a similar way to that of platelets or blood macrophages which can be physically linked to cancer cells, allowing them to travel together through the circulation[118]. Thus, single circulating tumor cells (CTC), which are no longer present in an immunosuppressive environment, may be targeted again by immunosurveillance. In this regard, the interaction of circulating cancer cells with PMNL may protect them from cell death, thereby overcoming immunosurveillance[119]. The journey of CTC ends upon integrin-dependent arrest on the endothelium, followed by extravasation. In this regard, systemic inflammation enhances attachment of CTC to endothelial cells, and this process is governed by neutrophil-dependent upregulation of adhesion molecules[120]. Thus, the production of high levels of proinflammatory cytokines by the PMNL can upregulate expression of certain adhesion molecules on endothelial cells and thereby increase the probability of metastatic cell attachment and potentialize the passage of tumor cells from the circulation into the extracellular space and then to develop micrometastases[90,105].

CONCLUSIONS AND PERSPECTIVES IN THERAPIES TARGETING NEUTROPHILS

Different proinflammatory molecules and inflammatory cells have been suggested to be potential candidate targets for therapeutic strategies for cancer[99,121,122]. One study has shown that different drugs that prevent inflammation can inhibit carcinogenesis[123].

The role of PMNL in the onset and progression of digestive carcinoma, in particular those occurring in inflammatory bowel diseases, is complex. However, recent studies highlight new aspects of the pathophysiology of the PMNL-epithelial cells interaction, in particular, the effect of ROS release by activated PMNL on digestive epithelial cells at the molecular level or the effect of different TAN on tumor progression. Interestingly, these novel findings on the role of PMNL in the initiation and progression of carcinogenesis open up therapeutic avenues for the treatment of digestive cancers[124]. It is noteworthy that immunotherapy against cancer has been explored as a coadjuvant and has been based mostly on the properties of the adaptative immune system (i.e. B and T lymphocytes, dendritic cells) and of some components of the innate system (macrophages, NK cells, or complement proteins)[125,126]. PMNL have been rarely considered as a weapon against cancer. However, studies highlighting the anti-tumor efficacy of PMNL have been published. For example, suppression of the secreted protein acidic and rich in cystein, which is associated with the capacity of tumor cells to migrate and invade tissues, in malignant cells, led to the promotion of PMNL recruitment and induced tumor rejection[127]. However, the mode of action of PMNL that leads to the killing of tumor cells is not fully understood. It probably depends on the maturation of PMNL since in an animal model of lung tumors, only a subpopulation of PMNL i.e. TAN2 had an anti-tumor effect[31]. PMNL produce cytotoxic agents such as proteases, ROS, and defensins, all of which can directly damage the target cells. However, the cytotoxic effect of PMNL on tumors is greatly enhanced in the presence of target-specific antibodies. Finally, another strong argument for the anti-cancer effect of PMNL comes from studies using animal models in which tumor cells were genetically engineered to release immunoregulatory molecules (cytokines and chemokines). These molecules did not affect the proliferation of the tumors directly, but activated a host immune reaction that was strong enough to overcome their oncogenic capacity. For instance, G-CSF-releasing colon adenocarcinoma cells were found to lose their tumorigenic activity through the massive attraction of PMNL to the tumor injection site[128]. These PMNL distinguished between G-CSF-producing and nonproducing cancer cells. Moreover, tumor inhibition *in vivo* was accompanied by intimate physical contact between PMNL and G-CSF-producing tumor cells[129]. However, future research should be done in order to better target the different subpopulations of TAN, since only one population of PMNL would have an anti-tumor effect and should be considered.

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TOPIC HIGHLIGHT

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Leptin in hepatocellular carcinoma

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Abstract

The risk factors for hepatocellular carcinoma (HCC) development have been established, and include chronic hepatitis B and C, heavy alcohol consumption, and aflatoxins. In fact, 5%-30% of patients with HCC still lack a readily identifiable risk factor. It has been reported that the majority of "cryptogenic" HCC may be attributed to nonalcoholic fatty liver disease, the hepatic presentation of the metabolic syndrome (MS). Obesity is associated with the development of the MS. Recently, adipose tissue has been considered as an endocrine organ because of its capacity to secrete a variety of cytokines, which are collectively known as the adipokines. Leptin, the product of the obese gene, is mainly produced by adipose tissue. Since leptin was first characterized in 1994, accumulated literature has demonstrated the involvement of this adipokine in several areas of human physiology. After binding to its receptor, leptin initiates a cascade of signaling events and subsequent cellular effects. In addition to being the regulatory mediator of energy homeostasis, several *in vitro* studies have demonstrated the fibrogenic role of leptin in the liver. Furthermore, the deregulated expression of leptin and its receptor have been demonstrated to be associated with a variety of metabolic disorders as well as human cancers. Most importantly, direct evidence supporting the inhibitory and/or activating role of leptin in the process of carcinogenesis and progression of human HCC has been accumulating rapidly. This review aims to provide important insights into the potential mechanisms of leptin in the development of HCC. Hopefully, further investigations will shed light on a new therapeutic target in HCC.

Key words: Adipokine; Hepatocellular carcinoma; Leptin; Liver cirrhosis; Metabolic syndrome; Obesity; Steatohepatitis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most com-

mon cancer worldwide and the third leading cause of cancer death[1]. Due to its high mortality, the annual fatality ratio is close to 1.0, indicating that the patients who develop HCC will die within 1 year[2]. Furthermore, recent data have shown that there are 662 000 deaths per year from liver cancer[3]. In addition, using the linked Surveillance, Epidemiology and End Results and Medicare dataset to estimate the annual direct and indirect costs associated with HCC, Lang *et al*[4] pointed out the considerable economic impact of HCC on the health care system in the United States. Thus, further understanding of the causation and potential mechanisms of HCC is urgently needed.

Recently, obesity has become a worldwide health issue, because it increases the risk for a variety of human diseases. It is said that the prevalence of obesity has increased substantially over the past decade in most industrialized countries, and a further increase is expected in the future[5]. The International Association for the Study of Obesity reported that approximately 40%-50% of men and 25%-35% of women in the EU were overweight [defined as a body mass index (BMI) between 25.0 and 29.9 kg/m^2], and an additional 15%-25% of men and 15%-25% of women were obese (BMI $\geqslant$ 30.0 kg/m^2)[6]. A similar observation was also found in the US population and the prevalence continues to increase despite all efforts to oppose it[7]. Diseases which have been associated with obesity include hypertension, type 2 diabetes, dyslipidemia and coronary heart disease[8,9]. Further evidence suggests that obesity is also a risk factor for certain types of cancer[10].

In spite of many well-defined risk factors for HCC [including hepatitis B virus (HBV), hepatitis C virus (HCV), and alcohol], Caldwell *et al*[11] have shown that 5%-30% of patients with HCC lack a readily identifiable risk factor. The majority of "cryptogenic" HCC in the United States is attributed to nonalcoholic fatty liver disease (NAFLD)[12]. In addition, a number of studies have observed an increased risk (1.5 to 4-fold) of HCC among obese individuals[13-15]. Therefore, experts have attempted to elucidate the possible events by which obesity might be linked to these diseases. Of note, it should be remembered that adipose tissue is central to the understanding of metabolic abnormalities associated with the development of obesity. In recent years, adipose tissue has been considered as an endocrine organ because of its capacity to secrete a variety of proteins with broad biological activities[16]. These proteins, collectively referred to as adipokines, play an important role in the physiology of adipose tissue, including food intake and nutrient metabolism, insulin sensitivity, stress responses, reproduction, bone growth, and inflammation.

Leptin, the product of the obese (*ob*) gene, has undoubtedly been the most studied adipokine since this protein was first characterized by Zhang *et al*[17] in 1994. Leptin is best known as a regulator of food intake and energy expenditure *via* hypothalamic-mediated effects. It is currently appreciated that this adipokine has many additional effects, often as a consequence of direct peripheral actions. These include angiogenesis, hematopoiesis, lipid and carbohydrate metabolism and effects on the reproductive, cardiovascular and immune systems. More importantly, a recent study considered leptin as a fibrogenic factor in all types of chronic liver disease[18]. In addition, leptin has been demonstrated to be crucial in the progression of NAFLD, the hepatic presentation of the metabolic syndrome (MS), into liver fibrosis[19]. Thus, the aim of this review is to discuss the updated information on leptin and its receptor, the proinflammatory effects of leptin on chronic liver disease of different etiologies, and the potential impact of leptin on HCC progression. Hopefully, leptin will shed light on a new therapeutic target in HCC treatment.

LEPTIN - A VERSATILE ADIPOKINE

Leptin, the product of the *ob* gene, is mainly produced by adipose tissues and, to a lesser extent, by tissues such as the stomach, skeletal tissue and placenta[17]. Leptin is secreted into the blood stream in a circadian rhythm and proportional to body fat mass. Although leptin serves as a regulatory mediator between the brain and the periphery through modulating the hypothalamo-pituitary-adrenal (HPA) axis, its circulating level is also regulated by hormones secreted by the HPA system, including corticosteroids, prolactin, and insulin[20,21]. Furthermore, leptin expression can be negatively regulated by fasting, beta-adrenergic agonists and thiazolidinediones. In addition, leptin secretion is higher in females than in males for any given age and body fat mass, i.e. it is sexually dimorphic.

Leptin is known to regulate energy homeostasis[22]. However, leptin-deficient (*db*/*db*) mice are not only severely obese, but also have a number of abnormalities. Research efforts have since expanded to elucidate leptin's role in human physiology and have resulted in a fundamentally renewed understanding of its role in the regulation of neuroendocrine function, reproduction, gastroduodenal mucosa defense, and metabolism of bone[23]. Indeed, changes in plasma leptin concentrations or in leptin action have important and wide-ranging physiological implications.

Recently, mounting evidence has advocated leptin to have a regulatory function in immunity similar to the function of a pro-inflammatory cytokine. Several studies have found that circulating leptin levels increase during infection and inflammation, suggesting that leptin is part of the immune response and host defense mechanisms. Leptin levels are acutely increased by many acute phase factors, such as tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6, and during bacterial infection, or lipopolysaccharide (LPS) challenge[24]. Leptin acts on monocytes/macrophages by inducing eicosanoid synthesis, nitric oxide and several pro-inflammatory cytokines. Moreover, leptin induces chemotaxis of neutrophils and the release of oxygen radicals. The role of leptin in the innate and adaptive immune responses has also been reviewed recently[25].

LEPTIN RECEPTOR AND ITS SIGNALING PATHWAY

The leptin receptor (OBR), belongs to the class I cytokine

receptor family (which includes receptors for IL-6, IL-12 and prolactin), and exists in at least six alternatively spliced forms with cytoplasmic domains of different length, known as OBRa, OBRb, OBRc, OBRd, OBRe and OBRf. These receptors are membrane-spanning glycoproteins with fibronectin type Ⅲ domains in the extracellular region and with a shared 200-amino-acid module containing four conserved cysteine residues and two membrane proximal cytokine-like binding motifs, Trp-Ser-Xaa-Trp-Ser[26]. Only the long form of the leptin receptor can signal intracellularly, whereas the short forms do not[27]. The short forms of the leptin receptor are expressed by several non-immune tissues and seem to mediate the transport and degradation of leptin. The long form of OBR, known as OBRb, is expressed by the central nervous system in areas that are responsible for the secretion of neuropeptides and neurotransmitters that regulate appetite, body weight and energy homeostasis[28]. Interestingly, OBRb is also expressed by endothelial cells, pancreatic β-cells, the ovary, CD34+ hematopoietic bone-marrow precursors, monocytes/macrophages, and T and B cells[25].

After binding leptin, OBRb initiates a cascade of signaling events. Foremost, the receptor-associated Janus-family tyrosine kinase 2 becomes activated by auto- or cross-phosphorylation, and subsequently tyrosine phosphorylates the cytoplasmic domain of the receptor[29]. At least three phosphorylated tyrosine residues in the cytoplasmic domain of OBRb function as docking sites for cytoplasmic adaptors: Tyr_{985}, Tyr_{1077} and Tyr_{1138}. Each of these phosphorylation sites lies in a unique amino acid motif, and each of these residues thus recruits a distinct set of downstream signaling proteins when phosphorylated.

In cultured cells, phosphorylated Tyr985 recruits the SRC homology 2 domain-containing phosphatase 2 to mediate the first step in the activation of the extracellular signal regulated kinase cascade[30]. Phosphorylated Tyr1138 recruits the signal transducer and activator of transcription-3 (STAT3), a latent transcription factor that then becomes phosphorylated, translocates to the nucleus, and mediates the regulation of gene expression[31]. Tyr1138→STAT3 signaling promotes the expression of SOCS3, as the afferent arm of a feedback loop that attenuates OBRb signaling[32]. The phosphorylation of Tyr1077 promotes the recruitment, tyrosine phosphorylation and transcriptional activation of STAT5, although Tyr1138 may also play a minor role in the regulation of STAT5 phosphorylation[33].

LEPTIN AND MS

Obesity, particularly abdominal obesity, is associated with resistance to the effects of insulin on peripheral glucose and fatty acid utilization, often leading to type 2 diabetes mellitus. Insulin resistance, the associated hyperinsulinemia and hyperglycemia, and the production of adipokines may lead to vascular endothelial dysfunction, an abnormal lipid profile, hypertension, and vascular inflammation, all of which promote the development of atherosclerotic cardiovascular disease (CVD). Therefore, the co-occurrence of metabolic risk factors for both type 2 diabetes and CVD (abdominal obesity, hyperglycemia, dyslipidemia, and hypertension) suggests the existence of a "MS"[34].

A study by Zimmet *et al*[35] reported the association of leptin with fasting insulin in several populations, raising the possibility that hyperleptinemia was an additional component of the MS, or perhaps even underlay the syndrome. Subsequent study also found that leptin was strongly, positively correlated with BMI, fasting insulin, and mean blood pressure after adjusting for age and sex irrespective of glucose tolerance status. Linear regression models indicated that leptin was associated with insulin sensitivity independent of age, BMI, waist/hip ratio, triglycerides, HDL cholesterol, and hypertension[36]. The associations between leptin and components of the MS (insulin, blood pressure, triglycerides), independent of obesity measures, suggest that leptin is more than a mere "iostat" or indicator of obesity. Thus, hyperleptinemia or leptin resistance may also be an important etiological component of the MS, either directly or *via* its influence in regulating insulin sensitivity.

ESTABLISHED RISK FACTORS OF HCC AND THEIR POTENTIAL LINKS TO LEPTIN

The established risk factors for HCC include HBV or HCV infection, alcohol intake, tobacco smoking, and aflatoxins. Their respective hepatocarcinogenesis and potential relationship with leptin will be reviewed in this section.

Chronic hepatitis B

To date, two major HBV-specific mechanisms have been indicated to contribute to HCC development. The first is the integration of the viral genome into the host chromosome causing cis-effects, resulting in loss of tumor suppressor gene functions, and/or activation of tumor-promoting genes[37]. The second mechanism involves the expression of trans-activating factors derived from the HBV genome, which have the potential to influence intracellular signal transduction pathways and alter host gene expression. A major player involved in this form of viral transactivation is the X protein (HBx). The HBx protein has been found to display pleiotropic functions and has been implicated in the malignant transformation of chronically-infected liver cells. By disrupting cellular gene expression, viral products such as HBx may modulate cellular growth, repair and death, consequently resulting in the transformation of hepatocytes to an oncogenic state[37,38].

Recent clinical data, which investigated serum leptin concentrations in patients with chronic viral hepatitis, indicated that cirrhotic patients due to HBV infection had significantly higher leptin levels compared to the controls, and serum leptin levels were associated with the stage of liver fibrosis. In addition, it was suggested that increased serum leptin levels might represent a negative prognostic factor for response to lamivudine monotherapy in patients

with chronic hepatitis B[39]. Another report, which evaluated the expression of leptin and ObR in patients with chronic viral hepatitis, found that the HBV patients expressed significantly lower ObR mRNA levels in peripheral blood mononuclear cells and had decreased serum leptin levels in comparison to the healthy controls. This implied involvement of the leptin system in the immunopathology of chronic viral hepatitis[40].

Chronic hepatitis C

Chronic HCV infection is characterized by inflammatory lesions in the liver, often accompanied by intrahepatic lipid accumulation (steatosis) and progressive fibrosis of variable degree, and long-term progression to cirrhosis and HCC[41]. The mechanisms underlying the progression of HCV infection to HCC still remain ill-defined. Unlike HBV, HCV does not integrate into its host genome and has a predominantly cytoplasmic life cycle[42]. Hepatocarcinogenesis of HCV, therefore, must involve several indirect mechanisms including the interplay between chronic inflammation, steatosis, fibrosis and oxidative stress and their pathological consequences. For example, the accumulation of oxidative stress and DNA damage in a setting of restricted cell cycle checkpoint control and/or accelerated cell division is thought to compromise gene and chromosome stability and to form the genomic basis for malignant transformation. Markers of intracellular oxidative stress have also been found to be increased in patients with chronic HCV infection as well as HCV core transgenic mice[43]. In addition, several HCV proteins have been shown to have direct oncogenic effects and to upregulate mitogenic processes[44]. In fact, direct interactions of the various HCV proteins with host cell factors have also been shown to lead to changes in cellular signaling cascades involved in regulation of cell metabolism and division and seem to be sufficient to induce hepatocarcinogenesis. Overall, it is thought that the synergism between chronic inflammation and direct virus-host cell interactions triggers the malignant transformation of hepatocytes. The requirement for such a synergism would also explain the slow "multi-step" transformation process that underlies human HCC development[45,46].

A previous study has demonstrated that chronic HCV infection could induce abnormal lipid accumulation in the liver[41]. Therefore, the association of leptin with this metabolic disorder has been reviewed. Some authors showed the link between leptin and obesity as well as hepatic steatosis development in patients with chronic HCV infection, however, this observation was not found in another report[47,48]. Likewise, higher leptin levels were shown to be associated with cirrhosis development due to chronic HCV infection[39], but not with their histological features[49]. These controversial results regarding the association of leptin with chronic HCV hepatitis needs further investigation.

Alcohol consumption

Chronic alcohol consumption has long been associated with the development of hepatic cirrhosis and subsequent HCC. Many deleterious effects of alcohol have been attributed to alcohol metabolism in hepatocytes. In general, alcohol is almost metabolized by alcohol dehydrogenase (ADH) located in the cytoplasm of hepatocytes. Acetaldehyde, which forms *via* ADH-dependent alcohol metabolism, is clearly of great significance during the initiation and progression of alcohol-related liver disease. It is said that acetaldehyde can alter the integrity of DNA in a variety of ways[50]. Chronic alcohol consumption can alter the balance of bacterial flora within the GI tract and the permeability to LPS[51]. The vascular architecture linking the GI tract to the liver, thus, leads to increased intrahepatic LPS levels and the stimulation/activation of the liver's resident macrophage kupffer cell (KC) population[52]. Once activated, KCs synthesize and release a range of proinflammatory cytokines, which can act in both an autocrine and paracrine manner to further activate KCs or neighboring cell populations[53,54]. The activation of KCs and associated cytokine release may affect hepatic responsiveness to alcohol in several different ways. For example, hepatic stellate cells (HSCs) can undergo rapid activation in the presence of hepatic insult including increases in proinflammatory cytokines, oxidative stress, and/or levels of hepatotoxins[55]. A central mechanism underlying ethanol-induced activation of HSCs is dependent on the generation of reactive oxygen species[56].

As mentioned above, chronic alcohol consumption activates KCs and subsequently releases a number of proinflammatory cytokines. A study from India, investigating the effect of exogenous leptin and/or ethanol on the secretion of TNF-α, IL-6 and transforming growth factor (TGF)-β1 both *in vivo* and *in vitro*, found that leptin could downregulate ethanol-induced secretion of proinflammatory cytokines and growth factor[57]. This implies that leptin could be useful in preventing the damage produced by ethanol, which might be of therapeutic interest.

Aflatoxins

Ecological studies in the 1970s and 1980s first reported correlations between aflatoxin levels in crops or food and HCC rates[58]. Aflatoxin B1 (AFB1) is the most common and potent of the aflatoxins. In areas of high aflatoxin exposure, up to 50% of HCC patients have been shown to harbor a specific AGG to AGT point mutation in codon 249 of the TP53 tumor suppressor gene (codon 249^{ser} mutation)[59,60]. Interestingly, one prospective epidemiological study has shown a more than multiplicative interaction between HBV and aflatoxins in terms of HCC risk[61]. A number of potential mechanisms have been mentioned, for example, the fixation of AFB1-induced mutations in the presence of liver regeneration and hyperplasia induced by chronic HBV infection, and predisposition of HBV-infected hepatocytes to aflatoxin-induced DNA damage[62]. On the other hand, one recent study also suggested that aflatoxin-albumin adducts were associated with more advanced liver disease in individuals infected with HCV[63].

In addition to being a strong carcinogen, AFB1 is also

known to evoke a decrease in food intake and body weight gain. In one *in vitro* experiment, it was demonstrated that AFB1 had a weak effect on adipocytes, but no significant influence on leptin release[64]. Another animal model found that AFB1 could decrease food intake and body weight, and significantly depress serum leptin levels[65].

NAFLD: A NEW RISK FACTOR FOR HCC

NAFLD is a spectrum of disorders ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis. It is believed to account for up to 90% of cases of elevated liver function tests in patients without an identifiable cause of liver disease (e.g. viral hepatitis, alcohol, inherited liver disease, and medications)[66]. Given the fact that patients with NASH can enter a final cirrhotic pathway similar to that in patients with alcoholic cirrhosis or in patients suffering from chronic hepatitis B or C, it is not surprising that NASH appears to be a new risk factor for HCC[67].

A close relationship between NFLD, obesity and the MS

Obesity is found in 30%-100% of subjects with NAFLD. In obese persons, steatosis is 4.6-fold higher than in normal weight persons[66]. Clinical, epidemiological and biochemical data strongly support the concept that NAFLD is the hepatic manifestation of the MS. According to Kotronen *et al*[68], 90% of individuals with NAFLD have at least one risk factor for MS, and 33% have all the features of MS. In addition, liver fat content is significantly increased in subjects with the MS as compared with those without the syndrome, independently of age, gender, and BMI. The presence of multiple metabolic disorders such as diabetes mellitus, obesity, dyslipidemia and hypertension is associated with a potentially progressive, severe liver disease[69].

Pathogenesis of NAFLD

Insulin resistance, oxidative stress, and an inflammatory cascade are believed to play integral roles in the pathogenesis and progression of NAFLD[70]. In insulin-resistant states, adipose and muscle cells preferentially oxidize lipids, resulting in the release of FFA. FFA can then be taken up by the liver, resulting in steatosis. Animal studies show that FFA, once released from muscle and adipose cells, can be incorporated into triglycerides in the liver or undergo oxidation in mitochondria, peroxisomes or microsomes. Oxidized by-products are harmful adducts that can cause liver injury, resulting in subsequent fibrosis[71]. Lipid peroxidation and oxidative stress result in increased production of hydroxynonenal and malondialdehyde that upregulate liver fibrosis *via* activation of stellate cells and result in increased production of TGF-β[72].

Recently, scientists have focused on the role of KCs in the pathogenesis of NAFLD. KCs are the resident macrophages of the liver and function in both innate and adaptive immunity as active phagocytosing agents and antigen-presenting cells (*via* toll-like receptors, among others) to T-cells. While inactivation of KCs is associated with NAFLD and impaired hepatic regenerative capacity, elimination of resident KCs has been associated with improvement of NASH, suggesting that overactivation of a Kupffer-cell-mediated immune response might underlie liver injury in NAFLD. It is thought that KC physiology becomes altered in the setting of increased hepatic lipid content possibly due to overcrowding of liver sinusoids resulting in prolonged exposure of KCs to antigens, reduced KC outflow, and a resulting sustained inflammatory response. Uncoupling proteins, are molecules that dissipate the proton gradient in the inner mitochondrial membrane and thereby reduce the energy needed for oxidative phosphorylation. Insufficient uncoupling protein production in KCs, possibly due to LPS-induced activity, might contribute to the pathogenesis of NAFLD[73].

Leptin in the pathogenesis of NAFLD

Leptin is thought to participate in the development of NAFLD. In animal models of NAFLD, leptin contributes to the development of insulin resistance and subsequently steatosis. Furthermore, in the context of liver insult, leptin has a proinflammatory role and is considered to be an essential mediator of liver fibrosis. In rats treated with carbon tetrachloride (CCl4), leptin injections have been shown to result in the increased expression of procollagen-I, TGFβ1 and smooth muscle actin, a marker of activated HSCs, and eventually to increased liver fibrosis[74].

In human studies, leptin levels were initially found to be significantly higher in 47 NASH patients than in 47 controls and correlated with the severity of hepatic steatosis but not to necroinflammation or fibrosis[75]. A subsequent study showed that leptin levels are significantly higher in NASH patients than in patients with chronic viral hepatitis and correlate with more severe fibrosis in univariate analysis[76]. However, another study failed to show any significant difference in leptin levels between NASH patients and controls or any independent association with liver fibrosis[77]. Thus, it is doubtful whether leptin is upregulated in patients with NASH and larger studies with a homogenous population and carefully matched healthy controls are needed. For the time being, leptin cannot be used as a noninvasive marker for the diagnosis of NASH.

LEPTIN PLAYS A FIBROGENIC ROLE IN THE LIVER

The development of fibrosis, which is critical for the progression of all chronic liver diseases, comprises a series of events, including inflammation, activation of fibrogenic myofibroblasts (e.g. HSCs), deposition of fibrillar extracellular matrix, and possibly neo-angiogenesis[78]. The data on the role of leptin in the regulation of these steps have been accumulating rapidly. Several *in vivo* studies, which evaluated the effect of leptin in animal models of chronic liver injury, including dietary steatohepatitis, bile duct ligation, and infection with eggs of *Schistosoma mansoni*, provided obvious support to the role of leptin as a critical mediator of fibrosis[79-81]. Importantly, when assessing

the response of ob/ob mice, the decreased fibrogenic response was reverted by supplementation with recombinant leptin. These findings suggest that leptin is a critical factor for the development of fibrogenesis in rodents.

Different cell types have been mentioned to participate in the response of leptin to liver injury, including KCs, sinusoidal endothelial cells and myofibroblast-like cells, which are derived from the activation of HSCs and from other mesenchymal cells. Ikejima *et al*[82] demonstrated that ObRb is expressed by sinusoidal endothelial cells and KCs, where exposure to recombinant leptin up-regulates the expression of TGF-β. A number of successive studies also indicated that HSCs express functional ObRb and are directly responsive to leptin. Expression of ObRb is low in quiescent HSCs, and increases during the activation process, suggesting that in activated HSCs the effects of leptin are amplified[83]. In addition, incubation of HSCs with recombinant leptin stimulates the expression of type I procollagen, potentiates the effects of TGF-b, and up-regulates expression of the tissue inhibitor of metalloproteinase-1, thus blocking collagen degradation[84,85].

A recently identified effect of leptin on fibrogenic cells is the induction of vascular endothelial growth factor (VEGF) *via* oxygen-independent activation of hypoxia-inducible factor 1a, a master switch of the angiogenic response[86]. These observations may potentially have an impact on liver fibrosis, as formation of new blood vessels is a key component of the wound-healing response and has been suggested to play a role in the irreversibility of established cirrhosis. Together, these observations suggest the fibrogenic role of leptin in the liver.

EVIDENCE REGARDING THE ASSOCIATION OF LEPTIN WITH HCC DEVELOPMENT

Yang *et al*[87] first explored whether obesity might increase the risk for HCC, and found that ob/ob mice developed liver hyperplasia at the earliest stage of NAFLD and eventually HCC. This observation raised the intriguing issue that obesity-related fatty liver, itself, might be a premalignant condition. A number of studies have attempted to elucidate the possible effects of leptin in HCC development. Wang *et al*[88], who investigated whether leptin might be involved in the etiogenesis of HCC in cirrhotic patients, found that increased serum leptin was significantly correlated with cirrhotic change, but not with HCC occurrence. This finding was consistent with previous studies, indicating the fibrogenic effect of leptin in the liver. Another study, which evaluated the expression of leptin and its receptor in HCC specimens and adjacent non-tumorous tissues, first pointed out the involvement of leptin in the carcinogenesis of HCC[89]. However, the authors suggested further investigations should be carried out to define the inhibitory and/or activating role of leptin in the process of carcinogenesis and progression of human HCC.

A recent study found that, without leptin signaling, neither fibrosis nor HCC developed in the rat NASH experimental model, suggesting that leptin might play a pivotal role in the progression of fibrogenesis and carcinogenesis in NASH[19]. Further *in vitro* assay demonstrated the necessity of leptin-mediated neo-vascularization coordinated with VEGF in this progression. The involvement of leptin/OBR in the angiogenesis of human HCC was also shown in a recent study[90]. Notably, one *in vitro* assay demonstrated the proliferative and anti-apoptotic effects of leptin in HCC cells *via* Janus kinase 2-linked signaling[91]. Taken together, these findings implied that leptin-induced effects implicated in HCC development seemed to be inhibitory.

In fact, several studies have reported the contradictory effects of leptin in HCC growth. Elinav *et al*[92] first showed that exogenous leptin significantly decreased tumor size and improved survival rate in a murine model of HCC. The authors further demonstrated that the majority of these leptin-induced inhibitory effects might be mediated by the induction of natural killer cell proliferation and activation. Moreover, Wang *et al*[93,94], who evaluated the expression of leptin and its receptor in HCC specimens by immunostaining, further correlated the expression profile with Ki-67 expression, intratumor MVD, as well as overall survival, provided clinical evidence on the prognostic roles of leptin and OBR in HCC patients. First, OBR expression was inversely correlated with vascular invasion of HCC. Furthermore, high leptin expression was associated with better survival in patients with HCC, treated postoperatively with medroxyprogesterone acetate, a synthetic variant of human progesterone. As a result, it was suggested that both high leptin and OBR expression in HCC tissues could predict better overall survival.

CONCLUSIONS AND FUTURE DIRECTIONS

Leptin has an increasingly crucial role in a variety of human metabolic disorders and cancers. This parallels the increasing prevalence of NAFLD in patients with HCC. This work reviews the updated information on leptin, including its receptor and related signaling pathway, and provides important insight into the association between leptin and the MS and NAFLD as well as well-known risk factors for HCC. Moreover, research studies have demonstrated that leptin can exert a fibrogenic effect in the liver. In addition, evidence regarding the direct link between leptin and HCC development has been accumulating rapidly. This demonstrates the potential of the leptin-mediated effects in the carcinogenesis and progression of HCC, however, there is ample room for further research on its inhibitory and/or activating role. In addition, the role of leptin in the response of HCC to hormonal therapy deserves further research.

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OBSERVATION

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Reproductive changes associated with celiac disease

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Abstract

Celiac disease is a mucosal disorder of the small intestine that may be triggered by dietary exposure to gluten in genetically-susceptible individuals. The disorder is often associated with diarrhea, malabsorption and weight loss along with other extra-intestinal complications. Reproductive changes have been described, including impaired fertility and adverse pregnancy outcomes possibly related to immune-mediated mechanisms or nutrient deficiency. Other possible pathogenetic factors that may alter placental function include maternal celiac disease autoantibodies binding to placental transglutaminase, and genetic mutations that may facilitate microthrombus formation. Reports noting activation during pregnancy or the puerperium may be important, and suggest that celiac disease may also be hypothetically precipitated by maternal exposure to one or more fetal antigens.

Key words: Celiac disease; Infertility; Pregnancy; Postpartum celiac disease; Fetal outcome

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INTRODUCTION

Celiac disease is an immune-mediated mucosal disorder primarily affecting the small intestine in genetically-susceptible individuals[1]. It may be triggered by dietary exposure to gluten, and frequently causes chronic diarrhea, malabsorption and weight loss[1]. In some patients, extra-intestinal or autoimmune changes may occur, e.g. hepatobiliary[2], neurological[3], or endocrine disorders, such as hypothyroidism and insulin-dependent diabetes in children[4]. Of particular interest has been the effect of celiac disease and its treatment on fertility and pregnancy[5]. In recent years, there has been an increased recognition of possible changes in male and female fertility in celiac disease as well as the potential for adverse outcomes in pregnancy and the post-partum period that may lead to miscarriages and premature low birth weight fetal deliveries (Table 1).

ALTERATIONS IN FEMALE FERTILITY

Celiac disease continues to be increasingly recognized as a clinically silent disorder with limited or few intestinal symptoms, such as mild diarrhea. Often, females with reproductive disorders or pregnancy complications have no overt symptoms, or at most, fatigue associated with iron-deficiency anemia. As a result, reduced fertility in females or changes that include delayed menarche, amenorrhea and early menopause may conceivably be the initial clinical feature that ultimately results in a diagnosis of celiac disease. As serological screening has resulted in an appreciation that celiac disease may occur in up to 1%-2% of the general population, it is not surprising that this disorder is more readily detected in young women of childbearing age. Indeed, young women are still the most common

Table 1 Reproductive changes in celiac disease

Altered female fertility	Delayed onset of menarche, amenorrhea, early menopause, recurrent abortions, reduced rates of pregnancy
Altered male fertility	Gonadal dysfunction, altered sperm morphology and motility, reduced sexual activity
Alterations in pregnancy	Repeated miscarriages, premature delivery an impaired fetal growth with low birth weight, abnormal placental function
Postpartum activation of celiac disease	Hormonal or immune changes

group diagnosed with celiac disease. If changes in fertility can be documented in celiac disease, these may reflect underlying autoimmune complications of celiac disease or the negative nutritional effects of untreated disease.

Although some early case reports noted a possible association between celiac disease and infertility[6,7], systematic investigations have been limited and the precise definition of infertility *per se* may vary between studies. An extensive serological evaluation of 150 women with infertility from Finland demonstrated an apparently increased rate of celiac disease (i.e. overall rate, 2.7%)[8]. Similar results were later reported in 99 couples from Northern Sardinia (i.e. 3.03% of females)[9], and later, using more modern serological screening methods [i.e. tissue transglutaminase (tTGA), endomysial (EMA) antibodies] in 192 Arab women with unexplained infertility from Israel (i.e. 2.65%)[10]. In all three of these studies, small bowel biopsies were positive if abnormal serological screening results were present. As in most screening studies, however, biopsies in the serologically-negative screened populations were not done. Moreover, other studies have suggested that the evidence may not be quite as strong for a definite association with celiac disease. In a report from another center in Finland[11], a higher frequency of celiac disease in women with infertility or recurrent miscarriage could not be defined. A Czech study showed increased seropositivity in women with infertility, but unfortunately, biopsies were not reported[12]. Finally, in a selected Italian cohort of infertile women undergoing assisted reproduction techniques, a statistically significant result was not achieved[13].

Delayed onset of menarche, amenorrhea, early menopause, recurrent abortions and reduced rates of pregnancy in celiac disease may reflect an impairment of fertility. In 74 celiac patients from the United Kingdom[5], the reproductive period was longer for those on a gluten-free diet compared to those not on a diet but maternal health was not seriously impaired. A lower incidence of spontaneous abortions in celiacs on a gluten-free diet was also recorded. Similar results were reported in an Italian study[14]. In consecutively diagnosed celiacs compared to age- and "sexual behavior"-matched healthy controls, there was a significant delay in the mean age of menarche in untreated celiac patients (13.5 years compared to 12.1 years). Amenorrhea and repeated abortions were more common in the celiac group, but onset of menopause did not significantly differ. Studies from Poland and Italy[15,16] also evaluated menarcheal age of celiac girls with reference to maternal menarcheal age. In one[15], menarcheal age of celiac girls appeared to be regulated by a gluten-free diet, while in the other[16], menarcheal age in celiac disease was not delayed, but was affected by maternal menarcheal age. A further evaluation from the United Kingdom[17] suggested that celiacs are subfertile with an increased incidence of stillbirths and perinatal deaths. However, after diagnosis of celiac disease and treatment with a gluten-free diet, some markers of infertility (e.g. miscarriage rates) may be corrected. Finally, in a study from Brazil[18], adherence to a gluten-free diet and resultant nutritional status was emphasized as an important and relevant factor in reproductive disorders developing in untreated celiac disease.

Nutritional studies in celiac disease during pregnancy are very limited. For example, zinc, selenium and folic acid deficiency have been noted in some studies[19-21], but most of these have been completed in children so that these studies do not appear to offer a definitive explanation for altered fertility in women during their reproductive years with untreated disease. Others have offered contradictory data in untreated celiac disease: reduced vitamins and trace elements were not evident, or significant malnutrition was not present[7-10]. In another report, evidence of poor vitamin status in celiacs despite a gluten free diet was reported[22], but this was contradicted by a detailed and more recent and important evaluation that documented histological recovery[23]. An alternative mechanism for reduced fertility may be immune-mediated, possibly by compromising placental function[24].

Clearly, further studies are needed to precisely define the role of altered absorption and resultant nutritional changes on female fertility in untreated celiac disease as well as the effects of a gluten-free diet, especially with restoration of normal nutritional status. In addition, immune-mediated changes in placental function need to be explored in celiac disease.

ALTERATIONS IN MALE FERTILITY

Studies estimating prevalence of male infertility in celiac disease have been rare. In an infertile couples study from Northern Sardinia[9], a single male out of 99 (or about 1%) tested positive for celiac antibodies, including EMA. Later the typical small bowel biopsy findings of untreated celiac disease were detected. Although the prevalence in a comparable control population was not provided, it is likely that an effective evaluation for infertility in a couple would best include assessment of both sexes for underlying celiac disease.

Early studies from the United Kingdom on male gonadal dysfunction described a reversible state of androgen resistance in celiac disease[25]. Later, in a further series of studies[26-29] on male gonadal function, consecutive males with celiac disease were evaluated and compared to males of similar age and nutritional status with Crohn's disease. Almost 20% of married celiacs had infertile marriages[26]. Semen analysis revealed marked abnormalities in sperm morphology and motility, similar to Crohn's disease, with sperm morphology apparently improving following removal of dietary gluten[26]. Others reported the presence

of oligospermia[27]. Specific nutrient deficiencies and detection of anti-sperm antibodies did not appear to be a factor in male infertility[26]. Plasma hormone levels were also determined[28]. Plasma testosterone and free testosterone index were increased while dihydrotestosterone was reduced. These hormone levels appeared to normalize, with an improved small bowel architecture on a gluten-free diet. Serum luteinizing hormone was also raised and interpreted to reflect androgen resistance. These endocrine changes, suggestive of androgen resistance and hypothalamic-pituitary dysfunction, were interpreted to be relatively specific to celiac disease, but an association with disordered spermatogenesis was not determined[28]. Further studies of gonadotropins were also performed[29]. Exaggerated gonadotrophin responses were apparently unrelated to plasma concentrations of testosterone, dihydrotestosterone, estradiol or the free testosterone index. Elevated prolactin levels were also noted but these were not related to impotence or infertility. These studies that suggested deranged pituitary regulation of gonadal function in celiac disease in males were hypothesized to be part of a wider disturbance of central regulatory mechanisms of endocrine function in celiac disease. Interestingly, less significant, but similar alterations in male sex hormone status occur in dermatitis herpetiformis[30], a skin disorder closely linked to celiac disease. These changes possibly also reflect, in part, the recognition of an autoimmune pituitary process associated with celiac disease and reported to be directly associated with an impairment in linear growth[31].

As many of these studies appeared almost three decades ago, it is striking that very little additional new, even descriptive, information on male infertility in celiac disease has appeared. However, two more recent Italian publications have explored sexual behavior[32,33]. In one study, sexual behaviors in treated and untreated celiac disease patients were examined using a questionnaire and compared to healthy controls[32]. Sexual satisfaction, including frequency of intercourse, was reduced in celiac patients, but improved after a year of treatment with a gluten-free diet. In the other[33], sexual habits appeared to be very different in celiacs who were never treated with a gluten-free diet.

ALTERATIONS IN PREGNANCY

Celiac disease, especially if untreated, appears to increase the risk of repeated miscarriages and premature deliveries, and impaired fetal growth with reduced birthweight[34]. In addition, adverse effects on the mother may also occur, as indicated by a recent German study which demonstrated that the rate of cesarean delivery was increased if the parents had celiac disease compared to other digestive disease controls as well as controls from eye or dental outpatient clinics[35].

In a case-control study from Italy that evaluated 94 untreated and 31 treated celiacs, the relative risks of either abortion or delivering a low birthweight baby were increased while the duration of breast feeding was significantly reduced[36]. All of these changes were apparently corrected with a gluten-free diet[36]. Higher incidences of either miscarriages or spontaneous abortions were also recorded from other centers located in different countries including Argentina, Italy and the United Kingdom[5,14,17,37].

Reduced birthweight and intrauterine growth retardation have also been recorded in several other studies from different European centers[38-41]. In one of these studies from Italy[39], the investigators also noted that celiac disease was a more common disorder than most of the diseases normally screened for in pregnant women in their healthcare facility. In another of these studies[40], undiagnosed maternal celiac disease appeared to be a far greater risk factor than diagnosed celiac disease, but in a subsequent report, undiagnosed celiac disease was not associated with an unfavorable outcome of pregnancy[42]. Other studies noted that paternal celiac disease did not appear to be a risk for an adverse pregnancy outcome[43,44].

Mechanisms involved in the impairment of pregnancy outcome in celiac disease have been explored to a limited degree. The placentas in mothers affected with celiac disease appear to be abnormal. In particular, tTG expression and apoptosis were reported to be increased in trophoblast cells using immunohistochemical analysis and *in situ* hybridization methods, suggesting a possible mechanism of injury in both the fetal and maternal parts of the placenta[45]. Others have noted that maternal celiac disease autoantibodies bind directly to the syncytiotrophoblast and inhibit placental tissue transglutaminase activity suggesting a possible mechanism for compromised placental function[24]. Furthermore, recent studies in celiac women on the role of genetic prothrombin variants in early pregnancy loss suggested that the 4G variant of the plasminogen activator inhibitor-1 gene may predispose to miscarriage[46]. Early pregnancy loss could conceivably relate to some alteration in coagulation affecting placental or fetal microvascular function. Additional studies are needed to further explore and elucidate these mechanisms.

FURTHER CONCLUDING ISSUES

Interestingly, delivery of a preterm infant has been conversely linked to later definition of underlying celiac disease in the parent, particularly underlying maternal celiac disease. In 905 preterm infants born in Lombardy, Italy, 1714 parents (868 women, 846 men) were screened for celiac disease using EMA and tTGA (followed by duodenal biopsy confirmation). In these, a higher prevalence of celiac disease in mothers of low birthweight infants was defined[47]. Thus, selective screening for celiac disease may be useful. However, the potential value and cost-effectiveness of screening for celiac disease in all women of reproductive age has not been fully determined.

Also intriguing is the repeated, but uncommon, definition of underlying maternal celiac disease following delivery in the puerperium[48-51]. Activation of celiac disease during the puerperium has been hypothesized to be related to immunologic or hormonal factors, or both[48]. Occasionally, anemia is evident during the prior pregnancy[49] or the presentation may be acute[50] leading to speculation

that activation of celiac disease may result from maternal exposure to fetal antigens[50]. In addition, presentation of underlying celiac disease may not be evident during an initial pregnancy, but may only appear after a second or later pregnancy[51]. Additional studies that explore the specific immunological changes occurring during the post-partum period may shed additional light on immune-mediated changes that occur in unrecognized celiac disease.

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ORIGINAL ARTICLE

Toll-like receptor 9 gene mutations and polymorphisms in Japanese ulcerative colitis patients

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Abstract

AIM: To investigated gene mutations and polymorphisms of *TLR9* in Japanese ulcerative colitis (UC) patients.

METHODS: Three single nucleotide polymorphisms (SNPs) in *TLR9* were identified in healthy controls, and were assessed in 48 UC patients and 47 healthy controls. Control subjects were matched for age, sex and date of blood sampling from among a subgroup of participants.

RESULTS: *TLR9* -1486CC, 1174GG and 2848AA increase the risk of UC [odds ratio (OR) 2.64, 95% confidence interval (95% CI): 1.73-6.53, P = 0.042], and *TLR9* -1486TT, 1174AA and 2848GG decrease the risk of UC (OR 0.30, 95% CI: 0.10-0.94, P = 0.039), although there were no correlations between SNPs and disease phenotype or *TLR9* mRNA expression.

CONCLUSION: *TLR9* polymorphisms are associated with increased susceptibility to UC.

INTRODUCTION

Inflammatory bowel diseases (IBDs), which include ulcerative colitis (UC) and Crohn's disease (CD), are chronic inflammatory disorders of the digestive tract. Although the pathogenesis of IBDs is complex and remains unclear, it has been suggested that immunologic, environmental and genetic factors contribute to their etiology[1]. Both clinical studies of IBD and studies of animal colitis models implicate luminal bacteria as necessary for initiating and perpetuating intestinal inflammation[2,3].

Among several candidate IBD-related chromosomal regions and genes, the caspase recruitment domain 15 (*CARD15*) gene coding for the nucleotide oligomerization domain 2 (*NOD2*) gene was identified as having the strongest linkage for CD susceptibility[4,5]. *NOD2/CARD15* is an intracytoplasmic receptor that binds bacterial peptidoglycan-derived muramyl dipeptide. While three common variants of this gene (R702W, G908R and L1007fsinsC) have been reported in CD patients in Western countries, there were no variants found in Japanese patients[6].

Similarly to NOD2, Toll-like receptors (TLRs) are essential components of innate immunity that recognize microbial compounds from bacteria, fungi and viruses[7-9]. While TLR activation leads to transcription of inflammatory and immunoregulatory genes, recent studies have demonstrated that TLR signaling in intestinal sites can inhibit inflammatory responses and maintain colonic homeostasis[10-12]. *TLR9*, which recognizes unmethylated CpG DNA in bacteria and viruses[13,14], and its signaling pathway protect mice from experimental colitis through production of type I interferons (IFN)[15]. Therefore, we need to know whether *TLR9* is a protective molecule not only in a mouse model but also in human IBD.

As the relationships between UC and *TLR9* gene variation have not been reported to date, we focused on *TLR9* gene mutations or polymorphisms in UC. Our results demonstrate that *TLR9* genetic polymorphisms are associated with an increased risk of UC in the Japanese population.

Table 1 Basic subject data (mean ± SD) *n* (%)

	UC (*n* = 48)	Controls (*n* = 47)
Age (yr)	43.49 ± 15.48	41.21 ± 15.36
Sex (M/F)	21 (43.8)/27 (56.2)	21 (44.7)/26 (55.3)
Age at diagnosis (yr)	35.07 ± 14.39	
Disease duration (yr)	8.42 ± 8.49	
Familial disease	0 (0)	0 (0)
Disease location		
Proctitis	3 (6.25)	
Left colitis	27 (56.3)	
Right colitis	1 (2.1)	
Pancolitis	17 (35.4)	
Disease severity		
Light	17 (35.4)	
Mild	18 (37.5)	
Severe	13 (27.1)	
History of colectomy	5 (10.4)	
History of immunosuppressant	6 (12.5)	
History of colon cancer	3 (6.3)	

UC: Ulcerative colitis.

MATERIALS AND METHODS

Human subjects

After obtaining written informed consent based on the Declaration of Helsinki of the World Medical Association, 48 UC patients and 47 healthy controls were enrolled in this study. All subjects were Japanese, and visited our hospital between December 2005 and January 2007. UC diagnoses were confirmed by review of medical charts by clinicians. Standard clinical, endoscopic and histological criteria were used[16-18]. The following clinical characteristics were analyzed: sex, age at diagnosis, disease location and disease severity. Control subjects were matched for age, sex and date of blood sampling from a subgroup of participants who were healthy volunteers, free from IBD and malignant tumors. Clinical characteristics of these subjects are shown in Table 1. This study was approved by the Ethics Committee of Fukushima Medical University.

Quantitative real-time reverse transcription-polymerase chain reaction

Peripheral blood mononuclear cells (PBMC) from UC patients and healthy controls were isolated from heparinized blood by Ficoll Paque density-gradient centrifugation (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway). Total cellular RNA was extracted from isolated PBMC using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA).

cDNA was generated by using the HotStarTaq Master Mix Kit (QIAGEN GmbH, Hilden, Germany). Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using a TaqMan® fast universal PCR master mix (Applied Biosystems, Foster City, CA, USA) and on-demand gene-specific primers, assessed using StepOne™ real time PCR system (Applied Biosystems). The primers were as follows: *TLR9* (Hs00370913_s1) and *GAPDH* (Hs02786624_g1). Both primers were purchased from Applied Biosystems. Relative quantification was achieved by normalizing to the values of the *GAPDH* gene.

SNP discovery

Genomic DNA from UC patients and healthy controls was extracted from heparinized whole blood samples using the PUREGENE® DNA purification kit (Gentra Systems, Minneapolis, MN, USA).

All exons, introns and approximately 1500 bases of the 5'-flanking region and 1500 bases of the 3'-flanking region of the *TLR9* gene were amplified from the genomic DNA of one healthy control. The genomic sequences were based on the GenBank reference sequence NM_017442 and AC097637.2 for *TLR9*. The subject was selected at random. Thirteen *TLR9* gene segments were amplified by PCR with the primer pairs shown in Table 2, *Taq* DNA polymerase (QIAGEN GmbH, Hilden, Germany) and genomic DNA from a healthy control as a template. Amplified products were purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences UK Limited, Buckinghamshire, UK), and were subjected to direct sequencing with the GenomeLab™ DTCS-Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) on a CEQ2000 DNA Sequencer (Beckman Coulter). SNP offsets were calculated relative to the A base of the *TLR9* ATG start codon, such that SNPs in the promoter region upstream of the first intron have negative position numbers.

Genotyping

Three common SNPs (-1486T/C, 1174A/G, 2848G/A) were analyzed for genotyping. The -1486T/C SNP was genotyped by restriction fragment length polymorphism (RFLP). Briefly, genomic DNA fragments containing -1486T/C SNP were amplified by PCR. PCR products were digested with the restriction enzyme Afl II (TAKARA SHUZO Co., Ltd., Shiga, Japan), run on a 3% aga-

Table 2 Primers used in polymerase chain reaction for genotyping *Toll-like receptor 9* polymorphisms and three single nucleotide polymorphisms

Fragment (product)	Forward	Reverse
TLR9 polymorphisms		
-2109 to -1293 (817 bp)	5'-CCAAGGGACTCTGGGAAAG-3'	5'-CATGTCACCCTCTCAACAGGG-3'
-1626 to -1095 (532 bp)	5'-CAGCCTTCACTCAGAAATACCC-3'	5'-GGCCAACAAGGCCCTATG-3'
-1296 to -553 (662 bp)	5'-CATGGGAGCAGAGACATAATG-3'	5'-GCCAGGGTGTAGCTTGA-3'
-795 to -134 (662 bp)	5'-GAGTCTCCTCACCTAGATCAG-3'	5'-TATACCAGCCTAGTAGC-3'
79 to 962 (884 bp)	5'-CTGCAAGCAACAGTGACGG-3'	5'-AGCTTTCACTTAACCAATCCC-3'
-393 to 1698 (2092 bp)	5'-TACCCGCTACTGGTGCTATC-3'	5'-TGGCAGAGTCTAGCATCAGG-3'
996 to 1687 (692 bp)	5'-CTGGTTCTGAAGCCTAATTC-3'	5'-AGCATGAGGATGTTGGTATGG-3'
976 to 1899 (924 bp)	5'-CTGGATTCTAGGTCTCAGTCC-3'	5'-CTGGATTCTAGGTCTCAGTCC-3'
1540 to 2368 (829 bp)	5'-CCTGCCACATGACCATCGAG-3'	5'-CCTGCCACATGACCATCGAG-3'
1812 to 2520 (709 bp)	5'-AACCTCACCCACCTGTCAC-3'	5'-AACCTCACCCACCTGTCAC-3'
2407 to 3350 (944 bp)	5'-TGCAGATGAACTTCATCAACC-3'	5'-GCTGTTGCAGCTGACATC-3'
3267 to 4057 (791 bp)	5'-CAGGAAACCAGCTGAAGG-3'	5'-CAGGAAACCAGCTGAAGG-3'
3867 to 4701 (835 bp)	5'-GACTGGGTGTACAACGAGCTT-3'	5'-TTCTGCATGGGAAAGGTAGG-3'
SNP		
-1486	5'-CAGCCTTCACTCAGAAATACCC-3'	5'-GGCCAACAAGGCCCTATG-3'
1174	5'-CTGGTTCTGAAGCCTAATTC-3'	5'-AGCATGAGGATGTTGGTATGG-3'
2848	5'-TGCAGATGAACTTCATCAACC-3'	5'-GCTGTTGCAGCTGACATC-3'

TLR9: Toll-like receptor 9; SNP: Single nucleotide polymorphism.

Table 3 Allele frequencies in ulcerative colitis cases and disease-free controls for *Toll-like receptor 9* single nucleotide polymorphisms at -1486, 1174, 2848

SNP[1]	Allele	UC (n = 48)	Controls (n = 47)	dbSNP[2]	JSNP[3]
-1486	T/C	0.344/0.656[a]	0.532/0.468	0.647/0.353	ND
1174	A/G	0.344/0.656[a]	0.532/0.468	0.512/0.488	0.517/0.483
2848	G/A	0.354/0.646[a]	0.543/0.457	0.481/0.519	0.517/0.483
					[a]P = 0.013

[1]Positions were calculated taking the A of the Toll-like receptor 9 (TLR9) ATG start codon as position 1 based on Genbank Accession No. NM_017442; [2]dbSNP public database: http://www.ncbi.nlm.nih.gov/SNP. [3]JSNP is a database of Japanese SNPs: http://snp.ims.u-tokyo.ac.jp/index_ja.html; [a]P value by Fisher's Exact test, cases *vs* controls. SNP: Single nucleotide polymorphisms; UC: Ulcerative colitis; ND: No data.

rose gel and subsequently stained with ethidium bromide to visualize the bands. The other two SNPs (1174A/G and 2848G/A) were confirmed by direct sequencing. The primers used for PCR or sequencing are shown in Table 2.

Statistical analysis

Statistical analyses were performed using SPSS version 11.0.1 and SNPAlyze version 6.0 (Dynacom Co., Ltd. Yokohama, Japan). Cases and controls were compared using Fisher's exact test (Tables 3-6) for categorical items. Simple logistic regression models were used to analyze Genotype-Diplotype associations. The results are expressed as odds ratio (OR) with corresponding 95% confidence intervals (95% CI). The level of significance was set at 5%. We examined Lewontin's D' (|D'|) and the linkage disequilibrium coefficient r^2 between each pair of SNPs using the expectation-maximization (EM) algorithm in SNPAlyze software.

RESULTS

TLR9 mRNA expression

In order to confirm whether there were differences in *TLR9* expression, we performed quantitative RT-PCR of *TLR9* using PBMC from UC patients and healthy controls. There were no significant differences in *TLR9* mRNA expression (Figure 1).

SNPs and their allele frequencies

We sequenced both exons and approximately 1500 bases of the 5'- and 3'-regions of *TLR9* in one healthy control. Offsets for each SNP were calculated relative to the transcription start site of *TLR9* mRNA (GenBank Accession No. NM_017442). We identified three SNPs in the regions sequenced. Their locations in chromosome 3 and in the *TLR9* gene are illustrated in Figure 2. These SNPs were confirmed in dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) and JSNP (http://snp.ims.u-tokyo.ac.jp/index_ja.html). The remainder of the analysis will focus on these three relatively common SNPs.

We found a marginally significant association between the SNPs at -1486, 1174, 2848 and UC. Elevated risk for UC was associated with a C allele at -1486 (Fisher's exact test, P = 0.013), a G allele at 1174 (Fisher's exact test, P = 0.013), and an A allele at 2848 (Fisher's exact test, P = 0.013) (Table 3).

Table 4 Genotype frequencies in ulcerative colitis cases and disease-free controls for *Toll-like receptor 9* single nucleotide polymorphisms at -1486, 1174, 2848 *n* (%)

Genotype at *TLR9*		UC (*n* = 48)	Controls (*n* = 47)
-1486	CC	20 (41.7)[a]	10 (21.3)
	CT	23 (47.9)	24 (51.1)
	TT	5 (10.4)[a]	13 (27.7)
1174	GG	20 (41.7)[a]	10 (21.3)
	GA	23 (47.9)	24 (51.1)
	AA	5 (10.4)[a]	13 (27.7)
2848	AA	19 (39.6)[a]	9 (19.1)
	AG	24 (51.1)	25 (53.2)
	GG	5 (10.4)[a]	13 (27.7)
			[a]$P < 0.05$

[a]P value by Fisher's exact test, ulcerative colitis (UC) cases *vs* controls. TLR9: Toll-like receptor 9.

Table 5 Haplotype frequencies in ulcerative colitis cases and disease-free controls for *Toll-like receptor 9* single nucleotide polymorphisms at -1486, 1174, 2848 *n* (%)

Haplotype			UC (*n* = 96)	Controls (*n* = 94)
-1486	1174	2848		
C	G	A	62 (64.6)[a]	43 (45.7)
T	A	G	33 (34.4)[a]	50 (53.2)
C	G	G	1 (1.04)	1 (1.06)
				[a]$P < 0.05$

[a]P value by Fisher's exact test, ulcerative colitis (UC) cases *vs* controls.

Table 6 Diplotype frequencies in ulcerative colitis cases and disease-free controls for *Toll-like receptor 9* single nucleotide polymorphisms at -1486, 1174, 2848 *n* (%)

Diplotype			UC (*n* = 48)	Controls (*n* = 47)
-1486	1174	2848		
CC	GG	AA	19 (39.6)[a]	9 (19.1)
CC	GG	AG	1 (2.1)	1 (2.1)
CT	GA	AG	23 (47.9)	24 (51.1)
TT	AA	GG	5 (10.4)[a]	13 (27.7)
				[a]$P < 0.05$

[a]P value by Fisher's exact test, ulcerative colitis (UC) cases *vs* controls.

Genotype frequencies

We then analyzed genotype frequencies between UC patients and healthy controls. As shown in Table 4, a pattern of increased prevalence of CC genotypes at -1486 (Fisher's exact test, $P = 0.047$), GG genotypes at 1174 (Fisher's exact test, $P = 0.047$), AA genotypes at 2848 (Fisher's exact test, $P = 0.042$), and a decreased prevalence of TT at -1486 (Fisher's exact test, $P = 0.039$), AA genotypes at 1174 (Fisher's exact test, $P = 0.039$), GG genotypes at 2848 (Fisher's exact test, $P = 0.039$) was observed in UC patients, as compared with controls.

Pairwise linkage disequilibrium and estimation of haplotypes

We observed absolutely identical allele frequencies for the three common SNPs. Table 7 shows that three SNPs are in strong linkage disequilibrium (LD) in both UC patients and healthy controls. We also inferred three SNP haplotypes by using an expectation-maximization algorithm. The number of statistically inferred haplotypes is eight (i.e. 2^3). A total of three of the eight possible haplotypes are observed in Table 5. The usage of haplotype C-G-A was more frequent in UC patients when compared with healthy controls, and the difference was statistically significant. (Fisher's exact test, $P = 0.013$, Table 5).

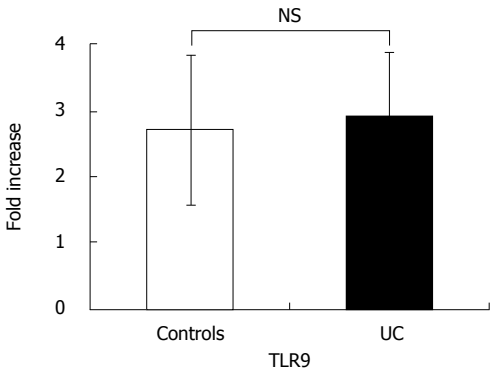

Figure 1 *Toll-like receptor 9* mRNA expression in peripheral blood mononuclear cells from ulcerative colitis patients and controls. Total RNA was prepared from peripheral blood mononuclear cells and Toll-like receptor 9 (TLR9) mRNA expression was analyzed by quantitative reverse transcriptase-polymerase chain reaction. There were no significant differences between ulcerative colitis (UC) patients and controls. NS: No significance.

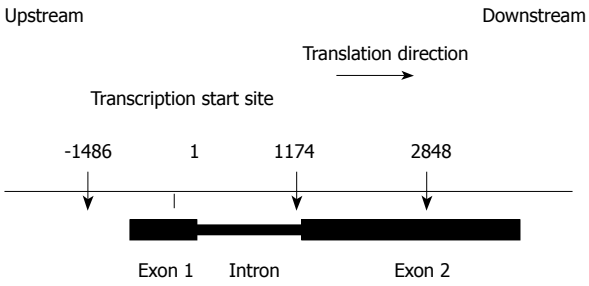

Figure 2 Gene structure and location of identified *Toll-like receptor 9* single nucleotide polymorphisms. We sequenced both exons and approximately 1500 bases of the 5'- and 3'-regions of *Toll-like receptor 9* in one healthy control. We identified three single nucleotide polymorphisms (-1486, 1174, 2848) in the regions sequenced.

Table 7 Linkage disequilibrium among single nucleotide polymorphisms of *Toll-like receptor 9* in ulcerative colitis cases and disease-free controls

SNPs		UC (*n* = 48)		Controls (*n* = 47)	
		|D′|	r^2	|D′|	r^2
-1486 T/C	1174 A/G	1	1	1	1
-1486 T/C	2848 G/A	1	0.9522	1	0.9581
1174 A/G	2848 G/A	1	0.9522	1	0.9581

Lewontin's D′ (|D′|) and the linkage disequilibrium coefficient r^2 between each pair of single nucleotide polymorphisms (SNPs) were examined by the expectation-maximization algorithm of SNPAlyze software. UC: Ulcerative colitis.

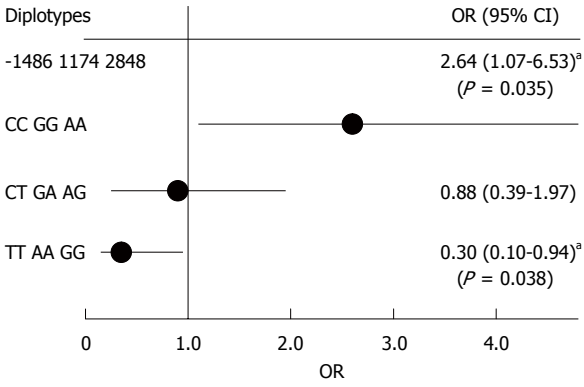

Figure 3 ***Toll-like receptor 9*** **gene diplotypes and disease sensitivity (expressed as odds ratios).** There was an increased risk of ulcerative colitis with the CCGGAA diplotype, and a decreased risk with the TTAAGG diplotype, as compared with controls. OR: Odds ratio; CI: Confidence interval. $^aP < 0.05$.

Diplotype frequencies

We next analyzed diplotype frequencies as pairs of haplotypes. Table 6 shows the distribution of three diplotype loci by disease status; an increased prevalence of CCGGAA diplotypes (Fisher's exact test, $P = 0.042$) and a decreased prevalence of TTAAGG diplotypes (Fisher's exact test, $P = 0.039$) was noted among UC cases, as compared with controls. Thus, we found an increased UC risk among CCGGAA diplotypes (OR 2.64, 95% CI: 1.73-6.53, $P = 0.042$) and a decreased UC risk among TTAAGG diplotypes (OR 0.30, 95% CI: 0.10-0.94, $P = 0.039$) (Figure 3). These data suggest that CCGGAA diplotypes are more susceptible to UC.

Genetic association with clinical phenotypes

In order to further examine the contribution of genetic variations in *TLR9* to UC phenotypes, we analyzed various clinical characteristics among UC patients: sex, age at diagnosis, disease location and disease severity. However, we did not observe any statistically significant correlations between disease status and diplotype, genotype or specific *TLR9* allele.

DISCUSSION

In this study, we found that genetic variations of *TLR9* are associated with an increased risk of UC in the Japanese population. *TLR9*, which is activated by unmethylated CpG DNA, triggers innate immune responses[19,20]. Although no variants have been found in Japanese patients, the *NOD2/CARD15* gene was found to indicate CD susceptibility in Western populations[5,6], suggesting that abnormal innate immune responses toward luminal bacteria are involved in pathogenesis of IBD. With regard to TLRs, previous reports have indicated that *TLR4* Asp299Gly polymorphism is associated with CD and UC in Caucasian populations[21]. Török *et al*[22] investigated possible associations between genetic variations in *TLR9* and IBD in the German population, but did not detect any associations between *TLR9* gene variations and UC susceptibility. Thus, as UC is a heterogeneous polygenic disease, association studies are expected to reveal various sets of susceptibility genes depending on the ethnic background of the study populations. Further genetic studies in different ethnic groups will resolve the role of the TLRs in UC susceptibility.

Associations between TLR9 and several autoimmune diseases or infectious diseases have been studied[23-26]. These reports have suggested that the -1237T/C SNP in *TLR9* is associated with an increased risk of allergic asthma[27], while 2848G/A was associated with myocardial infarction, deep vein thrombosis and chronic obstructive pulmonary disease[27], and 1635A/G was associated with HIV phenotype[28]. In addition, there were no associations between *TLR9* gene polymorphisms and susceptibility to systemic lupus erythematosus (SLE) and related phenotypes in Caucasian American subjects[23], but such an association was observed in a Japanese population[24]. These observations show that several candidate genes showing initial positive associations have generated negative findings in replication studies due to issues related to insufficient power or sample heterogeneity.

In this study, although relatively low numbers of subjects were enrolled, we observed statistically significant differences. According to the statistical methods used in this research, statistical differences in a category that has more than one subject indicate a reliable result. Moreover, we can expect to obtain the same results if more samples are added to the experiment. Therefore, we did not increase the power of this study, although more studies with large cohorts are necessary to characterize the role of *TLR9* SNPs in the etiology of UC.

We demonstrated that a C allele at -1486 (located in promoter region), a G allele at 1174 (located in intron 1) and an A allele at 2848 are associated with an increased risk of UC. SNPs in promoter regions potentially affect gene expression levels by altering the binding of gene transcription factors and SNPs in introns, thereby affecting mRNA splicing and/or enhancement of gene transcription. We were unable to identify down-regulation in *TLR9* expression in UC patients. Evaluating the relationship between the *TLR9* polymorphism and predefined clinical characteristics or biological markers also failed to demonstrate any impact on a particular UC phenotype. Taken together, these results indicate that *TLR9* polymorphism is associated with the development of disease itself, which is probably based on functional impairment of *TLR9*.

A recent study suggested that TLR9-triggered type-1 IFN protects mice from experimental colitis[15]. It also underscores the important protective role of type-1 IFN in intestinal homeostasis, suggesting that type-1 IFN produced by *TLR9* signaling affects the pathogenesis of IBD. Although further experiments are needed in order to identify the functional roles of *TLR9* polymorphism, either downstream molecules of the *TLR9* signaling pathway or cytokine production participating in the development of IBD is expected to be affected by these gene polymorphisms.

In conclusion, we identified an association between *TLR9* polymorphisms and UC in Japanese patients. Our findings indicate the importance of *TLR9* in the genetic

control of reactions to intestinal microbes in UC. Studying SNPs among the molecules involved in bacterial recognition will be essential to understanding the individual responses to bacterial components and to define the genetic background associated with risk of IBD.

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COMMENTS

Background

The pathogenesis of inflammatory bowel diseases (IBDs), which include ulcerative colitis (UC) and Crohn's disease (CD), is multi-factorial, involving susceptibility genes as well as immunological and environmental factors. Recent studies on IBD have provided some evidence that commensal bacteria play a key role in the pathogenesis of the disease, and that the Toll-like receptor (TLR) signaling pathway activated by commensal bacteria plays an important role in maintaining colonic homeostasis. The signaling pathway of *TLR9*, which recognizes unmethylated CpG DNA in bacteria and viruses, protects mice from experimental colitis. As the relationships between UC and *TLR9* gene variation have not been reported to date, in this report we focused on *TLR9* gene mutations or polymorphisms in UC.

Research frontiers

Among several candidate IBD-related chromosomal regions and genes, the caspase recruitment domain 15 (*CARD15*) gene coding for the nucleotide oligomerization domain 2 (*NOD2*) gene was identified as having the strongest linkage for CD susceptibility. Similarly to NOD2, TLRs are essential components of innate immunity that recognize microbial compounds from bacteria, fungi and viruses. While TLR activation leads to transcription of inflammatory and immunoregulatory genes, recent studies have demonstrated that TLR signaling in intestinal sites can inhibit inflammatory responses and maintain colonic homeostasis. A gene variation in *NOD2/CARD15* has been reported in CD patients in Western countries, but this variation has not been identified in Japanese CD patients.

Innovations and breakthroughs

As the relationships between UC and *TLR9* gene variation have not been reported to date, in this report we focused on *TLR9* gene mutations or polymorphisms in UC. Three single nucleotide polymorphisms (SNPs) in *TLR9* were identified in healthy controls, and were assessed in 48 UC patients. The authors found that *TLR9* -1486CC, 1174GG and 2848AA increase the risk of UC, and *TLR9* -1486TT, 1174AA and 2848GG decrease the risk of UC.

Applications

The authors' findings indicate the importance of *TLR9* in the genetic control of reactions to intestinal microbes in UC. Studying SNPs among the molecules involved in bacterial recognition will be essential to understanding the individual responses to bacterial components and to define the genetic background associated with risk of IBD.

Terminology

TLR9 is a mammalian Toll-like receptor homologue that appears to function as an innate immune pattern recognition protein for motifs that are far more common in bacterial than in mammalian DNA.

Peer review

It is interesting that the authors identified an association between *TLR9* polymorphisms and UC in Japanese patients. My main concern is the sample size (not too large) and the fact that the *P* values obtained are not extremely significant (almost marginally significant on some occasions). This may limit the validity of the present work.

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ORIGINAL ARTICLE

Retrorectal tumors in adults: Magnetic resonance imaging findings

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Abstract

AIM: To retrospectively evaluate the magnetic resonance imaging (MRI) features of adult retrorectal tumors and compare with histopathologic findings.

METHODS: MRI features of 21 patients with preoperative suspicion of retrorectal tumors were analyzed based on the histopathological and clinical data.

RESULTS: Fourteen benign cystic lesions appeared hypointense on T1-weighted images, and hyperintense on T2-weighted images with regular peripheral rim. Epidermoid or dermoid cysts were unilocular, and tailgut cysts were multilocular. Presence of intracystic intermediate signal intensity was observed in one case of tailgut cyst with a component of adenocarcinoma. Six solid tumors were malignant lesions and showed heterogeneous intensity on MRI. Mucinous adenocarcinomas showed high signal intensity on T2-weighted and mesh-like enhancing areas on fat-suppressed T2-weighted images. There was a fistula between the mass and anus with an internal opening in mucinous adenocarcinomas arising from anal fistula. Gastrointestinal stromal tumors displayed low signal intensity on T1-weighted images, and intermediate to high signal intensity on T2-weighted images. Central necrosis could be seen as a high signal on T2-weighted images.

CONCLUSION: MRI is a helpful technique to define the extent of the retrorectal tumor and its relationship to the surrounding structures, and also to demonstrate possible complications so as to choose the best surgical approach.

Key words: Retrorectal tumor; Presacral lesions; Magnetic resonance imaging; Congenital cyst; Malignant tumor; Diagnosis

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INTRODUCTION

Tumors occurring in the retrorectal space are extremely rare in the adult population, with an estimated incidence of 0.0025-0.014 in the large referral centers[1,2]. The ret-

rorectal space is a potential space that only becomes real when a mass grows within it. The boundaries of the retrorectal region include the posterior wall of the rectum anteriorly and the sacrum posteriorly. This space extends superiorly to peritoneal reflection and inferiorly to the rectosacral fascia and the supralevator space. Laterally, the area is bordered by the ureters and the iliac vessels and the sacral nerve roots.

This region contains the confluence of the embryologic hindgut, neuroectoderm, and the bony pelvis. As such, multiple different tissue types can give rise to retrorectal tumors. Retrorectal tumors may be classified as congenital, neurogenic, osseous or miscellaneous[3,4]. Two-thirds are congenital[2-4], caused by embryological sequestration, abnormalities in midline fusion and incomplete embryological regression. Cystic congenital lesions include epidermoid and dermoid cysts, tailgut cyst (also called mucus-secreting cyst), enterogenic cyst, teratoma, and teratocarcinoma. Neurogenic (including anterior sacral meningoceles), osseous and miscellaneous tumors each account for approximately 10% of retrorectal tumors[3]. Majority of these masses in adults are benign and asymptomatic, however, malignant tumors accounted for 21%[5]. Malignant transformation has also been documented in tailgut cysts and epidermoid cysts[6-8]. Symptoms include pain, change in bowel habit, difficulty with micturition, and neurological signs in the lower limb and perineum[3,4,9].

Retrorectal tumors are often found late and may be managed suboptimally. The current consensus is that the cardinal therapy for patients with retrorectal tumors is surgical[9-11]. Curative resection requires complete excision of the tumor, with an intact capsule for clinically well-circumscribed benign lesions and en bloc resection with clear resection margins for malignant tumors[9,11]. Accurate diagnosis of these conditions before operation is crucial because it can significantly alter clinical management. MRI is a useful technique to evaluate pelvic disorders because of its multiplanar imaging capability and its good soft tissue contrast[2,4,6,8,9,11]. We retrospectively evaluated the MRI features of retrorectal tumors in 21 patients and compared with pathological findings to further characterize the MR imaging findings encountered in retrorectal tumors.

MATERIALS AND METHODS

Patients

We reviewed the clinical and radiological findings in 21 patients (13 women, 8 men; age range, 16-74 years; mean age, 39.3 years) with preoperative suspicion of retrorectal tumor who were treated at our hospital between January 2006 and December 2008. Institutional review board permission was obtained for retrospective review without informed consent. MRI was performed in all patients with a standardized protocol. Data from clinic charts, hospital medical records, radiological and pathological reports of these patients were collected retrospectively. The clinical findings at presentation included rectal fullness (n = 7), low back pain (n = 2), constipation (n = 6), symptoms due to recurrent perirectal abscess and/or fistula (n = 5) and no apparent symptom (n = 1). Only two patients had postanal dimples. Digital examination of the rectum revealed a mass located posteriorly or laterally to the rectum in 20 patients. Laboratory tests demonstrated elevated carcino-embryonic antige (CEA) in two patients (2/21).

MR technique and parameters

All 21 patients underwent preoperative MRI. MR examinations were achieved on a 1.5-T unit using an eight-channel phased-array pelvic coil (Siemens, MR Magnetom Sonata, Germany). Neither bowel preparation nor Ⅳ contrast enhancement was performed. Before performing MR, the rectum was distended by a balloon filled with 60-80 mL saline. The MR examination was performed with the patient in a supine position. MR examination protocol included sagittal, axial, coronal T2-weighted images, and axial (n = 21) T1-weighted images. T2-weighted imaging (TR/TE, 4000/97; echo-train length 13, field of view 20 cm, 4 mm slice thickness, no interslice gap, 256 × 256 matrix) were performed in the sagittal plane (n = 21), axial plane (n = 21) and coronal plane (n = 9), and followed with fat-suppression in sagittal and axial plane. Imaging parameters for T1-weighted sequences were: TR/TE, 620/12; echo-train length 1, field of view 22 cm, 4 mm slice thickness, no interslice gap, 320 × 240 matrix. Overall acquisition time varied from 20 to 30 min.

Image review

Magnetic resonance images were reported by two experienced radiologists. Four features of the retrorectal tumor were assessed. (1) the tumor location and extent were defined by the most cephalad sacral vertebra involved; (2) tumor size was measured in the largest two dimensions; (3) tumor morphology was assessed by examining the internal signal characteristics and the tumor margin. A cystic tumor was diagnosed when the lesion displayed over 80% cystic elements and a solid tumor when the lesion showed more than 80% solid elements; the remainders were classified as heterogeneous tumors; and (4) the tumor margin was assessed as well-defined (a smooth or lobular contour without surface projections), irregular (with surface projections) or clearly invasive (the tumor breached an adjacent structure).

RESULTS

Eighteen patients underwent surgery for retrorectal tumor. One patient with a gastrointestinal stromal tumor (GIST) arising from the rectum received abdominal perineal resection (APR). Two patients, diagnosed as having mucinous adenocarcinoma arising from fistulae-in-ano, received radiochemotherapy because the patients refused surgery. Histopathological studies demonstrated 7 malignant tumors (2 mucinous adenocarcinomas arising from fistula, 2 gastrointestinal stromal tumors, 1 tailgut cyst

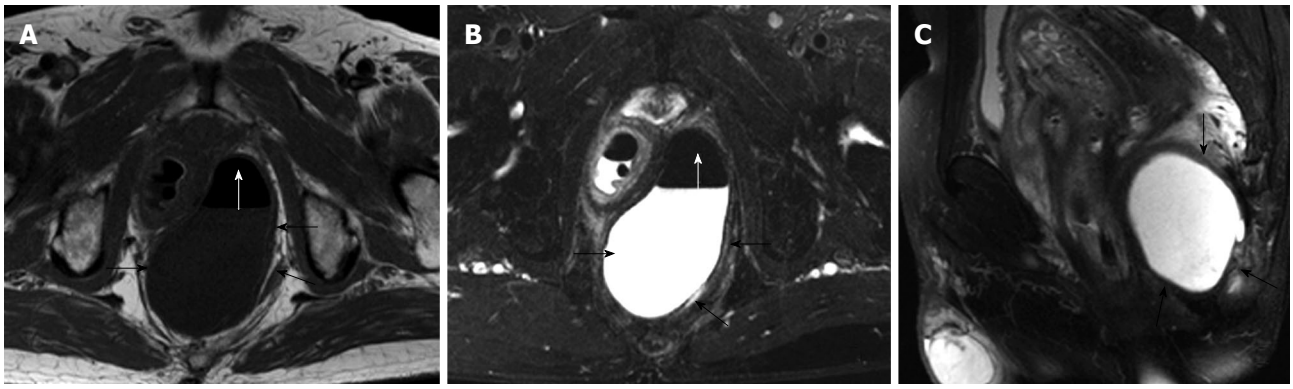

Figure 1 Dermoid cyst in a 63-year-old man, who was misdiagnosed as having abscess. Axial T1-weighted (A), T2-weighted fat-suppression (B) and Sagittal T2-weighted (C) magnetic resonance (MR) images show a thin regular peripheral rim (black arrows) circumscribing a retrorectal cyst and containing some air (white arrows). The cyst lesion extends to the left ischiorectal fossa, and is hypointense on T1-weighted (A) and hyperintense on T2-weighted MR images (B, C).

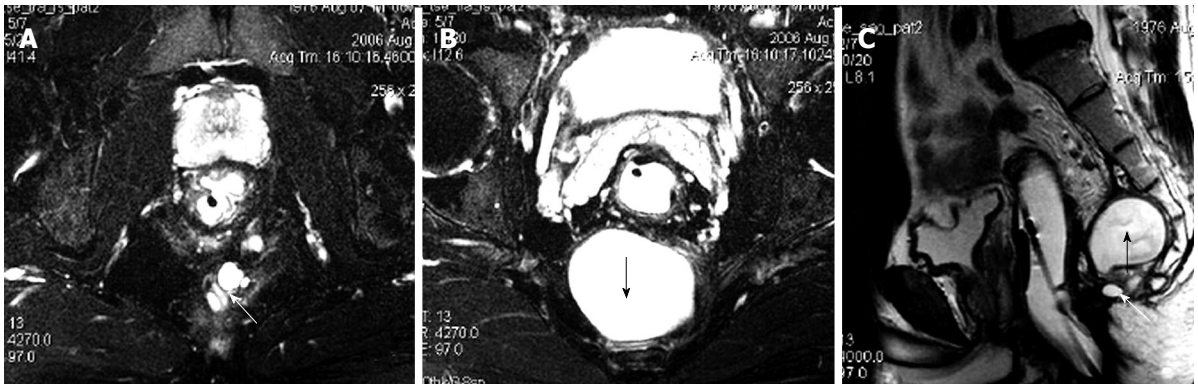

Figure 2 Tailgut cyst in a 30-year-old man. On T2-weighted fat-suppression (A, B) and Sagittal T2-weighted images (C), small cysts are composed to form a honeycomb (white arrows) adjacent to a main larger cyst (black arrows).

with component of mucinous adenocarcinoma, 1 adenocarcinoma of the anal duct, and 1 primary retrorectal adenocarcinoma) and 14 benign cysts (6 epidermoid cysts, 7 tailgut cysts and 1dermoid cyst). Patients with malignant tumors were significantly older than those with benign tumors (52.5 years *vs* 34.6 years). There was no gender difference in malignant tumors (male/female ratio, 4/3), and women comprised a larger proportion of the patients with benign cysts (male/female ratio, 3/11).

MRI demonstrated that benign cystic lesions were primarily located in the retrorectal space in 12 cases, with downward and lateral extension to ischiorectal fossa in two cases (Figure 1). The rectum appeared anterior or lateral in five patients. The mean largest diameter of the lesions was 4.8 cm (range 0.7-10 cm). Both epidermoid cysts and dermoid cysts were unilocular (Figure 1). There were numerous cysts in all tailgut cysts, shown as a large main cyst and associated with other smaller cysts in the same location (Figure 2). All the cystic lesions appeared hypointense on T1-weighted images, and hyperintense on T2-weighted images. As far as non-complicated cysts (10/14) were concerned, the cystic mass was well-circumscribed by a regular peripheral rim. The rim was hypointense on all sequences. The borders of the cystic lesions were irregular in 4 patients, who had an operative history with a misdiagnosis as having abscess or fistulae.

Heterogeneous tumor was found in one case of tailgut cyst with a component of adenocarcinoma (Figure 3). The rectum was compressed anteriorly but without evidence of invasion. The cystic portion appeared hypointense on T1-weighted images and hyperintense on T2-weighted images with irregular borders. The malignant portion presented as intermediate signal intensity on T2-weighted and fat-suppression with irregular margin.

The six solid tumors were malignant lesions confirmed by histopathology. Three patients were diagnosed as having mucinous adenocarcinoma, two as having mucinous adenocarcinomas arising from chronic perianal fistulae, and 1 as having adenocarcinoma of the anal duct (Figure 4). MRI showed a larger mass in the pelvis, extending to the ischiorectal fossa. The mucin, which forms the major tissue component of mucinous tumor, showed high signal intensity on T2-weighted fast SE images and mesh-like enhancing areas on fat-suppressed T2-weighted images (Figure 5). As for mucinous adenocarcinoma arising from fistula-in-ano, there was a fistula between the mass and the anus with an internal opening in anorectum (Figure 6). One was diagnosed as primary presacral adenocarcinoma after operation, and MRI identified a well-circumscribed 3.5 cm × 4 cm solid lesion with irregular margin in the

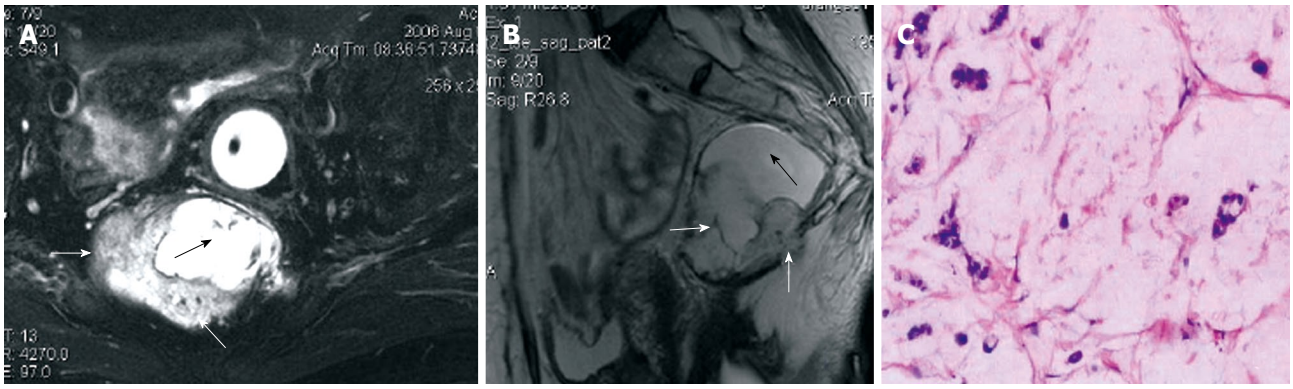

Figure 3 Tailgut cyst associated with mucinous adenocarcinoma in a 52-year-old woman. A, B: The rectum is compressed and shifted to the front but without evidence of invasion. The cystic portion (black arrows) is hyperintense, and the malignant portion (white arrows) presents as irregular margin with intermediate signal intensity on T2-weighted fat-suppression (A) and Sagittal T2-weighted images (B); C: Photomicrograph of the tumor shows a small cluster of atypical cells in stroma.

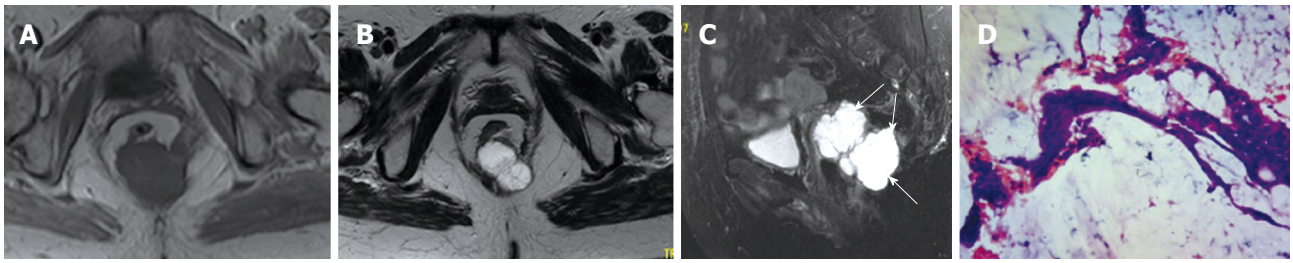

Figure 4 Adenocarcinoma of the anal duct in a 71-year-old woman. A, B: Axial T1-weighted magnetic resonance (MR) image shows a low signal mass with irregular marginal in the retrorectal space and high signal on T2-weighted MR image; C: The mucin, which forms the major tissue component of mucinous tumor, shows high signal intensity (white arrows) on fat-suppressed T2-weighted image; D: The gland shows irregular features with cellular hyperchromatin and the stroma shows marked desmoplasia.

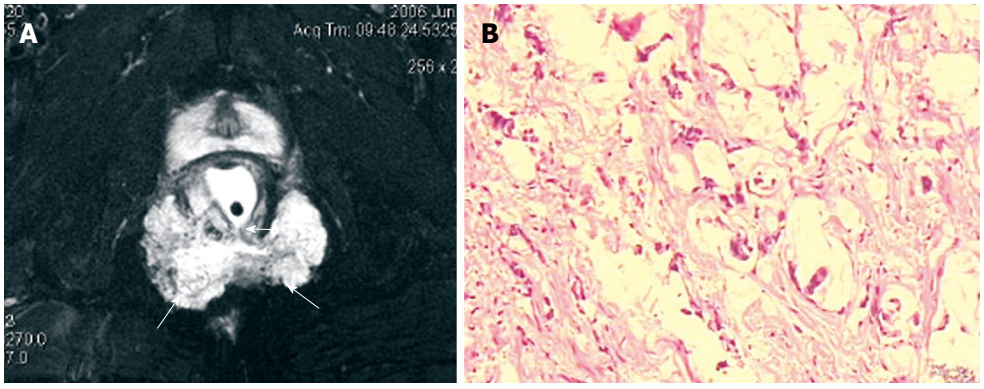

Figure 5 A 59-year-old man with mucinous adenocarcinoma caused by anal fistula. A: Axial fat-suppressed T2-weighted magnetic resonance image shows a horse-shoe mass with typical mesh-like enhancing areas (arrows). The mass has a internal fistula connected to the anorectum (arrows); B: Microscopy shows a single or small cluster of atypical cells floating in mucin pool and bundles of collagen with hyaline degeneration in stroma.

presacral space, not involving sacrum or rectum (Figure 7). The other two were diagnosed as gastrointestinal stromal tumors (GISTs), one appeared to arise from the rectum (Figure 8), and the other appeared in presacral space (Figure 9A and B). Tumors were larger than 5 cm in at least one transverse dimension. MRI showed low signal intensity on T1-weighted image, and heterogeneous signal intensity on T2-weighted image. We suspected a central necrosis based on the T2-weighted imaging with a high signal. In immunohistochemical studies, diffuse and strong immunoreactivity for CD34 and CD117 were seen throughout the tumors (Figure 9C and D). Conversely, the tumor cells were negative for both muscle markers (smooth muscle actin, desmin) and neural markers (S-100 protein, neuron specific enolase).

DISCUSSION

The boundaries of the presacral region include the posterior wall of the rectum and the sacrum. This space extends superiorly to peritoneal reflection and inferiorly to rectosacral fascia and the supralevator space. Laterally, the area is bordered by the ureters, the iliac vessels, and the sacral nerve roots. Because this area contains totipotential

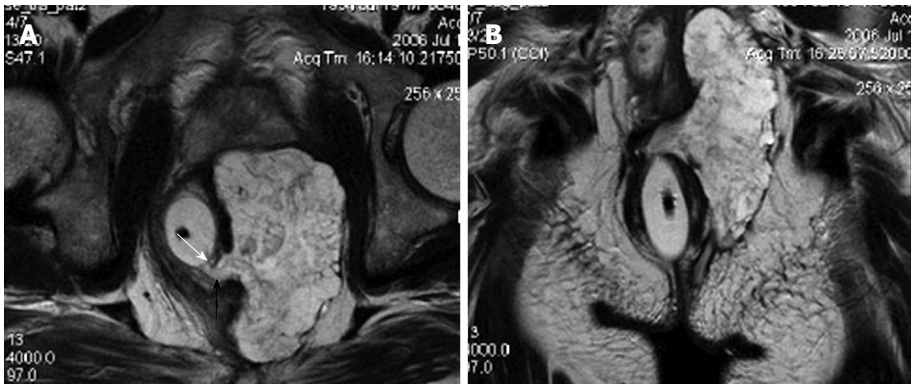

Figure 6 A 52-year-old man with mucinous adenocarcinoma caused by anal fistula. A, B: The mass displays heterogeneous intensity on T2-weighted magnetic resonance image. There is a fistula between the mass and the anus (black arrow) and an internal opening in anorectum (white arrow).

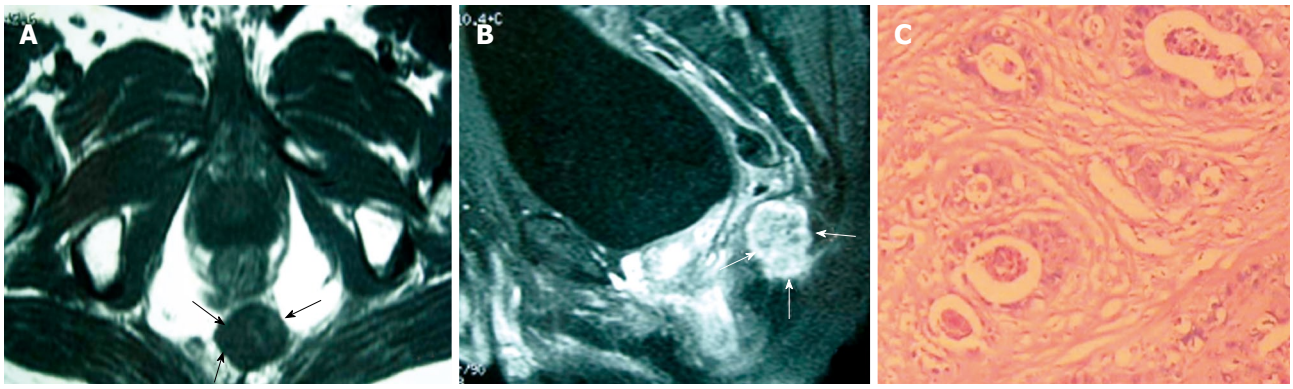

Figure 7 Primary retrorectal adenocarcinoma in a 33-year-old man. A, B: The mass displays low signal intensity, without rim (black arrows) on T1-weighted magnetic resonance (MR) image, and high signal intensity of the irregular border (white arrows) on fat-suppressed T2-weighted MR image; C: Microscopy shows irregular glands of variable sizes and atypical tumor cells. The stroma shows desmoplasia.

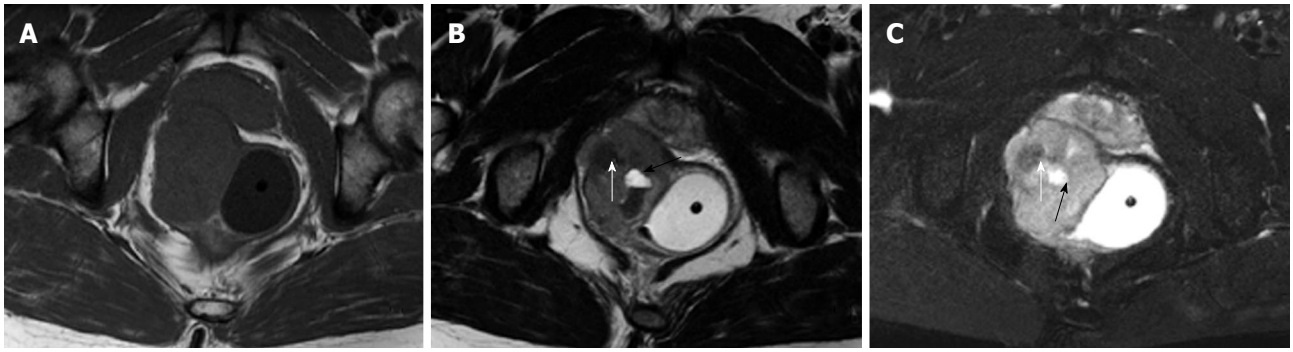

Figure 8 Gastrointestinal stromal tumors of rectum in a 44-year-old man. A well-circumscribed, smooth, intramural mass with exophytic growth expanded from the rectal wall and extended to the right ischiorectal fossa. The tumor displays low signal intensity on T1-weighted image (A), and high signal intensity on T2-weighted image (B, C). There are some areas of necrosis (black arrows) and fibrotic tissues (white arrows) in the tumor.

cells that differentiate into three germ cell layers, a multitude of tumor types may be encountered. Traditionally, these lesions are divided into congenital, inflammatory, neurogenic, osseous and miscellaneous types[3,4]. Clinical diagnosis may be delayed because of non-specific symptoms. Symptomatic patients typically complain of vague, longstanding pain in the perineum or low back, and change in bowel habit[1,5,8]. Because detection is often difficult and delayed, patients frequently present with tumors that have reached a considerable size and involve multiple organ systems, thus complicating their treatment. Singer *et al*[12] reported 7 patients who underwent an average of 4.7 invasive procedures or operations before the correct diagnosis of a retrorectal lesion was made.

The diagnosis and management of these tumors has evolved in recent years because of improved imaging modalities, especially the MRI. MRI is often used in diagnosing and managing the patients with presacral tumors, as it can provide excellent anatomic detail and soft-tissue contrast. Kim *et al*[13] asserted that for the evaluation of a

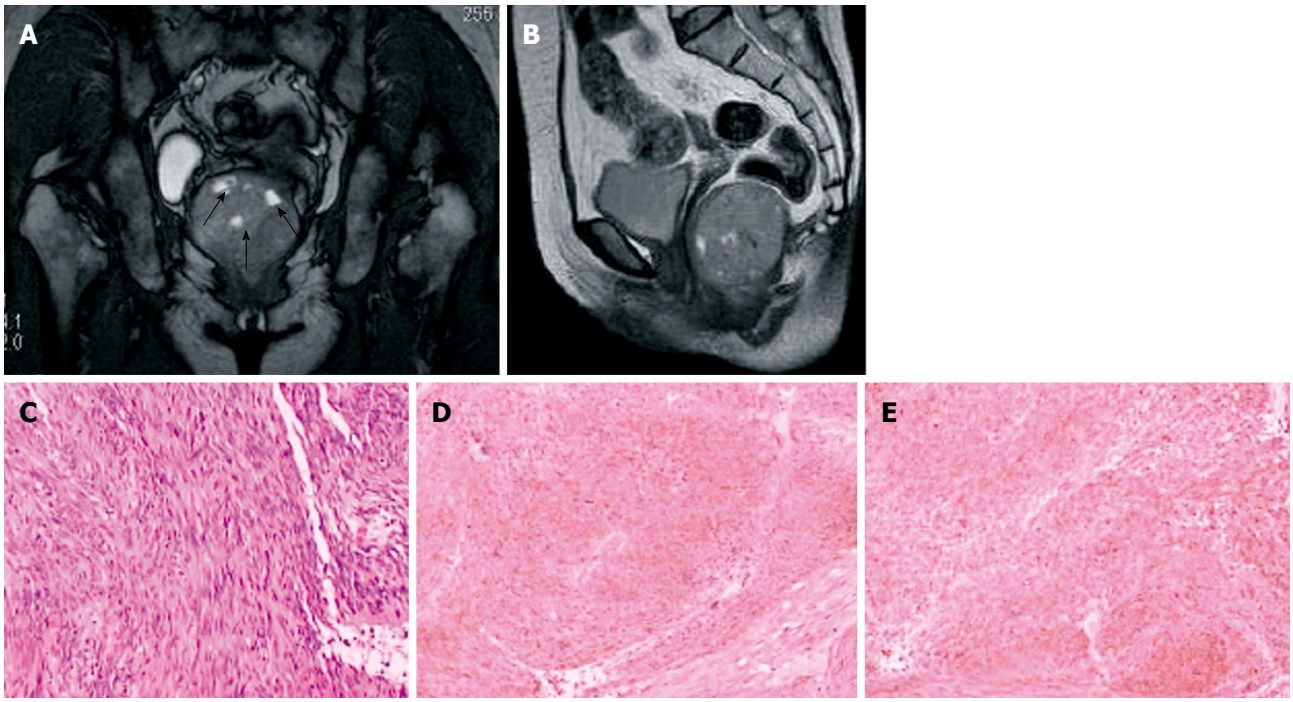

Figure 9 Retrorectal gastrointestinal stromal tumors in a 51-year-old woman. A, B: T2-weighted magnetic resonance images show a large retrorectal mass, in which some necrosis presents hyperintensity (black arrows); C: Histopathology shows spindle cells and cytoplasmic vacuoles; D, E: On immunohistochemical studies, diffuse and strong immunoreactivity for CD117 and CD34 are seen.

presacral mass, MRI has the advantage over CT of being able to offer multiplanar capabilities and good tissue contrast. MRI is a valuable tool for preoperative evaluation, imaging and characterizing lesions, estimating their extent and the risk of malignancy, distinguishing organ-confined disease from tumor spread into adjacent structures, and deciding upon the most appropriate intervention strategies and imaging follow-up requirements.

About half of the presacral tumors are congenital lesions, and most of them are developmental cysts (epidermoid, dermoid, tailgut cysts, and teratomas). On MRI, presacral cyst usually has low signal intensity on T1-weighted images and high signal intensity on T2-weighted images[8]. However, it may have high signal intensity on T1-weighted images due to presence of mucinous materials, high protein content, or hemorrhage in the cyst[6,8]. All of the presacral cysts in our cases were hypointense on T1-weighted images and hyperintense on T2-weighted images. Among the presacral cystic masses, epidermoid cyst, dermoid cyst, rectal duplication cyst, and meningocele are usually unilocular[14]. The presence of fat content on fat-saturated images is suggestive of a dermoid cyst[8]. Rectal duplication cysts often communicate with the rectal lumen and are anterior to the rectum. Anterior meningocele is a well-defined unilocular thin-walled, fluid-filled lesion of the retrorectal space with a stalk that may be seen communicating with the thecal sac. In contrast, tailgut cyst is usually multicystic[6]. In our study, we observed that epidermoid cysts and dermoid cysts appeared unilocular on MRI, and tailgut cysts appeared as a large cyst accompanied by small peripheral cysts. We believe that the unilocular or multilocular characteristics are very important because of the malignant potential of a tailgut cyst. A few cases of degenerated tailgut cysts have been documented in the literature[6,15-17]. The possibility of malignant transformation must be considered in the presence of heterogeneous tumor. Our findings, that a cystic lesion displayed heterogeneous signal intensity on T2-weighed MR images with markedly irregular wall, and the malignant portion presented as irregular margin with intermediate signal intensity on T2-weighted and fat-suppression, are consistent with what has been described in the literature[6,17].

MRI is useful in predicting whether a tumor is benign or malignant. A cystic tumor with a smooth, well-circumscribed margin and no features of invasion or enhancement with gadolinium is benign, and a heterogeneous tumor, or a solid tumor with an irregular margin, is usually malignant[9]. The six solid tumors were malignant confirmed by histopathology in this series. Two patients with adenocarcinoma arising from fistula-in-ano displayed presacral tumor. Several characteristic MRI findings may help diagnose mucinous adenocarcinoma arising from fistula-in-ano. Histopathologically, mucinous colorectal carcinomas comprise large pools of extracellular mucin lined by columns of malignant cells, cords and vessels, which give rise to a typical mesh-like internal structure[18]. The mucin, which forms the major tissue component of mucinous tumors, has high signal intensity on T2-weighted fast SE images. Two authors reported that a fistula between the mass and the anus is a characteristic finding of mucinous adenocarcinoma arising from fistula-in-ano on MRI[19,20]. Two patients showed a fistula tract between the mass and the anus in agreement with the previous reports.

GIST is a non-epithelial neoplasm arising from the

wall of the gastrointestinal tract. GIST is thought to originate from the interstitial cell of Cajal, an intestinal pacemaker cell[21]. GISTs are most often located in the stomach (39%-70%) and small intestine (20%-35%), whereas the colon and rectum (5%-12%) are less common locations[22-25]. Some GISTs primarily arise in the omentum, mesentery, or retroperitoneum and are unrelated to the tubular gastrointestinal tract. It is even rarer that GIST originates from presacral space. On MRI, solid portions of tumor typically show low signal intensity on T1-weighted images, intermediate to high signal intensity on T2-weighted images, and enhancement after administration of gadolinium[26]. The markedly high signal seen on T2-weighted MRI should be considered as a feature strongly indicating a diagnosis of GIST[27]. GISTs usually involve the muscularis propria of the gastrointestinal wall, so the characteristic image is that of a well-circumscribed, smooth, intramural mass with exophytic growth. The case with rectal GIST in this series presented as well-defined, eccentric mural mass that expanded the rectal wall and extended into the right ischiorectal fossa. As in GISTs at other locations, central areas of necrosis could be seen in our cases.

Adenocarcinomas of the presacral region are distinctly unusual. Most of the cases represent direct extension or metastatic spread from rectal cancer. Although malignant replacement of these cysts is possible, complete replacement of the cyst epithelium and other elements by adenocarcinoma has not been reported[5]. However, potential sources such as gastrointestinal, pancreatic and prostatic tissues were eliminated and no evidence of a developmental cyst was found histopathologically in this case. The tumor described in the present report should be regarded as a primary presacral adenocarcinoma, as reported by Zamir *et al*[5] and Puccio *et al*[28]. Pelvic MRI demonstrated a retrorectal heterogeneous solid lesion on both T1-weighted and T2-weighted images and a markedly high signal was seen on T2-weighted images after fat-saturation.

A further benefit of MRI being confirmed is whether routine preoperative biopsy is necessary[29]. Historically, the role of preoperative biopsy of retrorectal tumors has been a controversial topic in the general surgery. When preoperative MRI is available, the indications for biopsy can be limited to patients whose mass may represent metastatic disease or lymphoma[9]. The risk of a routine biopsy can, therefore, be avoided.

Retrorectal tumors can be best managed by surgery. Careful surgical planning is important by selecting appropriate approaches, such as an anterior approach (abdominal), posterior approach (perineal), or a combined abdominoperineal approach. MRI will help define the margins of resection and the relationship between the tumor and the sacral level. If the tumor is positioned below the midbody of S3, a perineal approach can be considered. All tumors that extend above S3 often require an abdominal or combined approach.

In conclusion, retrorectal tumor is a rare entity that is difficult to diagnose. Our series supports that MRI is a useful examination when a retrorectal tumor is suspected. Cystic lesions with a smooth wall on MRI are typically benign, whereas heterogeneous or solid tumors are usually malignant. However, final diagnosis should be based on the pathological examination after surgical resection. MRI is a helpful technique to define the extent of the tumor, its relationship to the surrounding structures and also to demonstrate possible complications in order to choose the best surgical approaches.

COMMENTS

Background

Tumors occurring in the retrorectal space are extremely rare in the adult population. Retrorectal tumors may be classified as congenital, neurogenic, osseous or miscellaneous types. Majority of these masses are benign and asymptomatic, however, malignant tumors accounted for 21%. Curative resection requires a complete excision of the tumor, with an intact capsule for clinically well-circumscribed benign lesions and en bloc resection with clear resection margins for malignant tumors. Accurate diagnosis of these conditions before operation is crucial because it can significantly alter clinical management.

Research frontiers

The diagnosis and management of retrorectal tumors have evolved in recent years because of improved imaging modalities, especial magnetic resonance imaging (MRI). MRI is a valuable tool for preoperative evaluation, imaging and characterizing lesions, estimating their extent and the risk of malignancy, distinguishing organ-confined disease from tumor spread into adjacent structures, and deciding upon the most appropriate intervention strategies and imaging follow-up requirements.

Innovations and breakthroughs

This series supports that MRI is a useful examination when a retrorectal tumor is suspected. Cystic lesions with a smooth wall on MRI are typically benign, whereas heterogeneous or solid tumors are usually malignant. MRI is a helpful technique to define the extent of the tumor, its relationship to the surrounding structures and also to demonstrate possible complications in order to choose the best surgical approach.

Applications

MRI is a useful technique to evaluate retrorectal tumors. It can provide optimal information about tumor location, size, extent, the risk of malignancy and strategy for therapeutic intervention in the patients with retrorectal tumor.

Terminology

Retrorectal space, also called presacral space, is a potential space limited anteriorly by the fascia propria of the rectum and posteriorly by Waldeyer's rectosacral fascia. Laterally, it is limited by the iliac vessels and the ureters and extends along the lateral ligaments of the rectum, communicating superiorly at the level of the peritoneal reflection with the retroperitoneal space.

Peer review

This article well documented the MRI features of retrorectal lesions, and this will interest our readers.

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BRIEF ARTICLE

Pro12Ala polymorphism of the peroxisome proliferator-activated receptor γ2 in patients with fatty liver diseases

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Abstract

AIM: To test the occurrence of the Pro12Ala mutation of the peroxisome proliferator-activated receptor-γ (PPARγ)2-gene in patients with non-alcoholic fatty liver disease (NAFLD) or alcoholic fatty liver disease (AFLD).

METHODS: DNA from a total of 622 specimens including 259 blood samples of healthy blood donors and 363 histologically categorized liver biopsies of patients with NAFLD (n = 263) and AFLD (n = 100) were analyzed by Real-time polymerase chain reaction using allele-specific probes.

RESULTS: In the NAFLD and the AFLD collective, 3% of the patients showed homozygous occurrence of the Ala12 PPARγ2-allele, differing from only 1.5% cases in the healthy population. In NAFLD patients, a high incidence of the Ala12 mutant was not associated with the progression of fatty liver disease. However, we observed a significantly higher risk (odds ratio = 2.50, CI: 1.05-5.90, P = 0.028) in AFLD patients carrying the mutated Ala12 allele to develop inflammatory alterations. The linkage of the malfunctioning Ala12-positive PPARγ2 isoform to an increased risk in patients with AFLD to develop severe steatohepatitis and fibrosis indicates a more prominent anti-inflammatory impact of PPARγ2 in progression of AFLD than of NAFLD.

CONCLUSION: In AFLD patients, the Pro12Ala single nuclear polymorphism should be studied more extensively in order to serve as a novel candidate in biomarker screening for improved prognosis.

Key words: Single nucleotide polymorphism; Peroxisome proliferator-activated receptor γ; Non-alcoholic steatohepatitis; Alcoholic steatohepatitis; Inflammation; Fibrosis; Hepatitis; Steatosis; Steatohepatitis

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INTRODUCTION

Fatty liver diseases are becoming a common cause of chronic liver diseases in the Western countries encountering in about 20% of the general adult and child population[1-5]. Excessive accumulation of triglycerides in hepatocytes occurring in etiologically diverse conditions causes hepatic steatosis characterized by more than 5%-10% fat stored either in macrovesicles or in microvesicles of hepatocytes[6,7]. Whereas in the past, regular and excess alcohol consumption was the most common reason for hepatic steatosis[8,9], fatty liver diseases are now most frequently associated with obesity, insulin resistance and type 2 diabetes due to an unbalanced and rich diet in industrial nations[3,5].

The spectrum of fatty liver diseases (FLD) independent of causative agents ranges from simple steatosis to steatohepatitis, which can progress to liver fibrosis ending up in cirrhosis or hepatocellular carcinoma[3,5,10-13]. Thus, 5%-10% of non-alcoholic fatty liver disease (NAFLD) patients with steatosis develop a steatohepatitis accompanied by a high risk of progression to fibrosis[3,4]. Although fatty liver diseases can have various causes, features of steatohepatitis in NAFLD and alcoholic fatty liver disease (AFLD) are difficult to distinguish histologically[6,7]. Both are characterized by foci of liver cell necrosis and lobular inflammatory infiltrates with polymorphonuclear leukocytes. Furthermore, the onset of steatohepatitis is accompanied by ballooned hepatocytes, often harboring Mallory's hyaline and megamitochondria or undergoing apoptosis[14]. Whereas steatosis seems to be more pronounced in non-alcoholic steatohepatitis (NASH) than in alcoholic steatohepatitis (ASH), features of necroinflammatory and cholestatic activity are more prominent in ASH liver biopsies[6]. Progression of steatohepatitis then results in pericellular fibrosis[6,7,15] involving myofibroblastic activation of sinusoidal hepatic stellate cells responsible for elevated extracellular matrix deposition[16].

Members of the peroxisome proliferator-activated receptors (PPAR) seem to play a key role in the pathophysiology of FLD by modulating increased glucose uptake and hepatic triglyceride accumulation, but also perform anti-inflammatory signals when steatohepatitis has occurred[17-19]. The PPAR family consists of PPARα, PPARγ, and PPARδ nuclear receptors, functioning as transcription factors, that mediate transcriptional response to insulin resulting in glucose uptake, increased fatty acid oxidation, lipogenesis and lipid storage, respectively[17]. Whereas the PPARα is highly present in hepatocytes, the splice variants PPARγ1 and 2 triggering adipogenesis are mainly expressed in adipose tissues and only to a minor extent in the liver. PPARγ increases the expression of genes that promote fatty acid storage, whereas it represses genes that induce lipolysis in adipocytes. In patients suffering from FLD, hepatic expression of PPARγ is shown to be involved in insulin sensitivity, triglyceride clearance and hepatic steatosis[20].

Due to its high impact as an insulin-sensitising transcription factor involved in adipogenesis and lipogenesis, the occurrence of single nucleotide polymorphisms (SNP) in the PPARγ gene was recently addressed by numerous reports studying subjects with insulin resistance, type 2 diabetes, arteriosclerosis, and hypertension[21-23]. A prevalent SNP association with impaired lipid homeostasis was observed in terms of the N-terminal proline alanine exchange (Pro12Ala) of the extra domain in the PPARγ2 variant. This PPARγ splice form includes 30 additional amino acids[24], which are responsible for a 5-6-fold increase of PPARγ's transcriptional activity. The Pro12Ala exchange in the activating extra region of the PPARγ2 is the result of a cytosine to guanine substitution in the PPARγ gene, as a consequence encoding the Ala-allele form with a heavily reduced function[23]. In several populations, the association of the Pro12Ala polymorphism with insulin-sensitivity, type 2 diabetes, obesity and adipositas have been shown[25-27]. However, the role of the Pro12Ala polymorphism of PPARγ gene in occurrence and progression of fatty liver diseases is not yet defined.

In the present study, we analyzed the frequency of the Pro12Ala polymorphism in the PPARγ gene by a highly sensitive LNA-probe based polymerase chain reaction (PCR) approach in a total of 622 subjects of a Caucasian population, suffering from fatty liver disease (n = 359) or being healthy blood donors (n = 263). In agreement with reports showing a high Ala allele prevalence in patients with impaired lipid metabolism in obese and adipose patients[26,28], in FLD patients the Ala allele also occurs more often than in the healthy control group. Interestingly, the interpretation and linkage of the allele frequency to histological evaluation and clinical data demonstrates a prominent risk in AFLD patients bearing the Ala allele to develop severe steatohepatitis and fibrosis. Furthermore, our data revealed for the first time a higher anti-inflammatory impact of PPARγ in progression of human AFLD than NAFLD.

MATERIALS AND METHODS

Patients, biopsies and liver disease classification

From a total of 622 cases, 259 blood samples and 363 biopsies were studied for occurrence of the Pro12Ala exchange in the PPARγ gene. Local research ethics guidelines were followed. We collected 363 cases from the files of the Department of Gastroenterology and Hepatology, University Hospital of Essen (GER) and the Institute for Pathology, University Hospital of Cologne (GER) according to their histological criteria of fatty liver disease (Table 1). 263 tissue specimens from patients were classified as NAFLD according to the clinical information about alcohol consumption (less than 20 g alcohol per day). One hundred specimens of patients who consume more than 20 g alcohol per day met the definition of AFLD as described by Neuschwander-Tetri *et al*[29]. Clinical data, such as GOT, GPT, and γGT, were compiled along with the state of diabetes. There was no appreciable difference between the mean age of AFLD (53.93 ± 10.63, range 20-81 years) and NAFLD (50.48 ± 15.25, range 16-80 years). All specimens, stained with haematoxylin and eosin (HE) and by the Gomori method for visualization of reticular fibers, were independently classified by three experienced liver pathologists (Hardt A, Drebber U, Dienes HP), according to

Table 1 Scoring according to the histological features described by Kleiner *et al*[15]

Definition	Score
Steatosis	
Grade	
< 5%	0
5%-33%	1
33%-66%	2
> 66 %	3
Localization	
Zone 3	0
Zone 2	1
Zone 1	2
Azonal	3
Panacinar	4
Type	
Macrovesicles	In %
Microvesicles	In %
Mixed	
Inflammation	
Lobular	
No foci	0
< 2 foci	1
2-4 foci	2
> 4 foci	3
Portal	
No inflammation	0
Minimal	1
Mild	2
Moderate	3
Severe	4
Fibrosis	
None	0
Mild/moderate	1
Periportal or perisinusoidal	2
Bridging fibrosis	3
Cirrhosis	4
Liver cell damage	
Ballooning	
None	0
Moderate	1
Severe	2
Mallory bodies	
None	0
Moderate	1
Severe	2

the histological score described by Kleiner *et al*[15] (Table 1). Additionally, 259 DNA extracts from blood samples of healthy blood donors were taken as references for local gene distribution.

Automatic DNA extraction from formalin fixed and paraffin embedded biopsies

Extraction of DNA from 363 formalin fixed and paraffin embedded (FFPE) biopsies was performed from three 7 μm-microtome sections after deparaffinization and proteinase K treatment, as previously described[30]. Then, DNA was purified by means of magnetic bead technology (FormaPure™ Kit of Agentcourt, Beverly MA, USA). All DNA purification steps were carried out by the BioMek FX laboratory automatic workstation (Beckman Coulter, USA) according to the work file and recommendations of Agentcourt.

Furthermore, DNA from 259 blood samples was prepared by the robotic workstation using the Genfind™ Kit of Agentcourt according to the manufacturer's instructions.

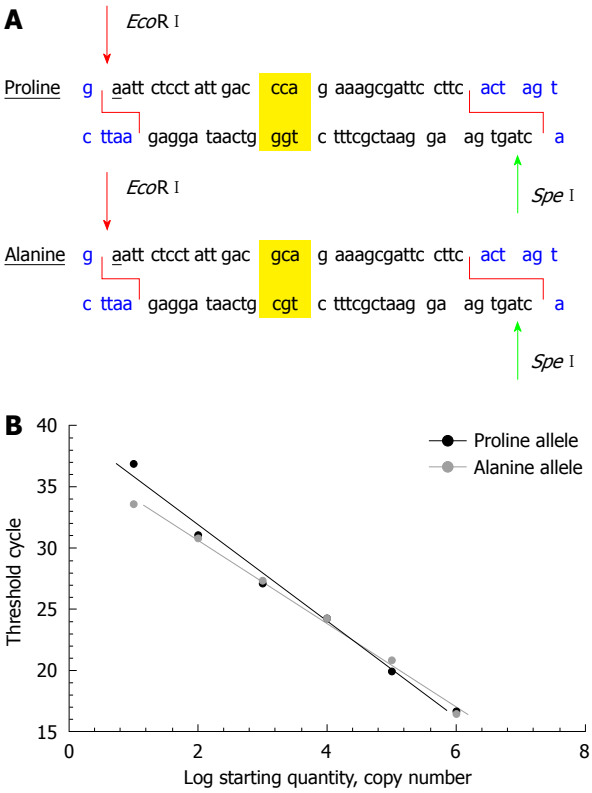

Figure 1 Cloning strategy and validation of a reference system. A: Cloning strategy for the generation of a reference system. The Pro12Ala mutation locus of the peroxisome proliferator-activated receptor-γ gene was synthesized as the proline or the alanine encoding oligonucleotides sequence. The proline or the alanine codon is indicated in yellow. The chemically synthesized oligonucleotide dimers, flanked by the overhangs of the *Eco*R I and the *Spe* I restriction sites (red/green), respectively, were used for insertion into pBluescript SKII plasmids; B: Real-time polymerase chain reaction (PCR) of the Pro12Ala locus depending on different copy numbers of reference sequences. Real-time PCR was performed using the LNA probes (Table 2) specific for the proline encoding sequence or the alanine encoding allele, respectively. 10^6, 10^5, 10^4, 10^3, 10^2, 10 copies of the plasmid reference sequences encoding either the Ala12 or the Pro12 locus were each diluted in 10 ng salmon sperm DNA and used for LNA probe based real-time PCR assays. Up to 10 copies per ng total DNA of both reference sequences were efficiently detected by the corresponding LNA probe labelled either by Hex or Fam fluorochrome.

Cloning of reference sequences into pBluescript

For construction of a reference system with sequences of the Pro12Ala locus (rs1805192) carrying either the mutation or the wild type sequence of PPARγ2 gene we used oligonucleotides comprising the proline or alanine encoding sequences (Figure 1). These oligonucleotides were dimerized in 150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L Tris pH 7.4, creating *Eco*R I and *Spe* I compatible overhangs and inserted into pBluescript SKII (+) (Stratagene, Texas, USA) by the respective restriction sites (Figure 1).

Allelic discrimination of the PPARγ2 by real-time PCR

The Pro12Ala exchange of the PPARγ gene (rs1801282) was examined in DNA from normal blood donors and patients by a Taqman probe associated real-time PCR. Genomic DNA was amplified by real-time PCR in a total vol-

Table 2 Real-time polymerase chain reaction primers and probes

Name	Oligonucleotide sequence	PCR application
Plas A primer	5'-CCGCTCTAGAACTAGTGAAGGAA-3'	Reference DNA
Plas S primer	5'-ACTCACTATAGGGCGAATTGG-3'	
PPARγ A primer	5'-TTACCTTGTGATATGTTTGCAGAC A-3'	Target DNA
PPARγ mis primer	5'-GTTATGGGTGAAACTCTTGGAGA-3'	
TM LNA probe wt	5'-6FAM-CTATTGACCCAGAAAGC--BHQ1	Target and reference DNA
TM LNA probe mut	5'-YAK-CTATTGACGCAGAAAGC--BHQ1	

Bold letters indicate the single nuclear polymorphism and underlined letters indicate the insertion of locked nucleotides (LNA). PPARγ: Peroxisome proliferator-activated receptor-γ; PCR: Polymerase chain reaction.

ume of 10 μL using the Eppendorf MasterMix (Hamburg, Germany) and 0.3 μL of each primer (10 μmol/L). The Plas A and Plas S primer set was used for amplification of reference plasmids; the PPARγ A and PPARγ mis primer set was used for genomic DNA samples (Table 2).

Allelic discrimination was achieved by adding 0.4 μL of 2.5 μmol/L LNA probes (TIB Molbiol, Berlin, Germany) recognizing the wild type and the mutant variant of the Pro12Ala locus of the PPARγ2 gene (Table 2). In parallel to the allelic Pro12Ala discrimination, plasmid reference sequences diluted from 10^5 to 10 copies in herring's sperm DNA (1 ng/mL) were applied to all assays as positive controls. Amplification and analyses were accomplished by the following cycling conditions using a MX3000P qPCR System of Stratagene (Texas, USA): initial denaturation at 95℃ for 2 min, following 50 cycles 95℃ for denaturation, 55℃ for annealing, 65℃ for extension, each step lasting 20 s.

Statistical analysis

Pro12Ala distribution was evaluated using the SPSS software 17 of IBM® (Chicago, USA). Significance of cross-classification was calculated by the Fisher's exact test. Odds ratios were used to describe the risk of disease progression.

RESULTS

Prominent occurence of the PPARγ alanine variant in patients with fatty liver disease

In order to detect the Ala12Pro polymorphism in patients with fatty liver disease we established an assay using locked nucleotide acid (LNA) probes for allelic discrimination. For this purpose, both variants of the Ala12Pro locus were cloned (Figure 1A) and the sequences, encoding either the proline or the alanine variant, were used as reference sequences for efficient allele detection and discrimination. The LNA probe hybridization assay linked to real-time PCR efficiently detected both, the proline wild type and the alanine mutated variant of the PPARγ gene (Figure 1B). Up to 10 copies of each reference sequence were successfully proven. In addition to the high sensitivity, application of the reference sequences attested that the LNA probe based PCR assay was highly specific, enabling the differentiation of the alanine and the proline encoding sequence.

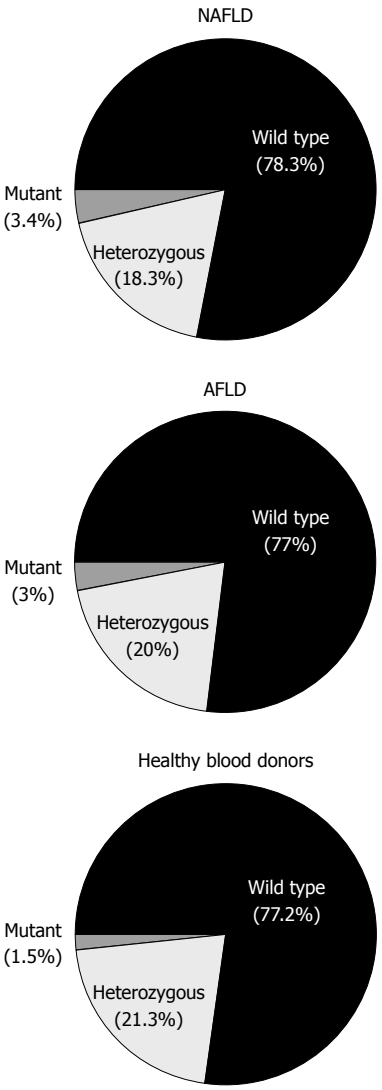

Figure 2 Frequency of the Pro12Ala polymorphism in patients with non-alcoholic fatty liver disease (*n* = 263), alcoholic fatty liver disease (*n* = 100) and in healthy blood donors (*n* = 259). The wild type allele, which is the Pro allele, is indicated in grey, the heterozygous genotype (Pro/Ala) in black and the homozygous Ala/Ala mutant in pale-grey. Genotype analyses revealed a higher prevalence of the homozygous Ala/Ala genotype in non-alcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease (AFLD) patients.

In the 259 healthy blood donors the assay accounted for 1.5% homozygous variants carrying the alanine encoding sequence (Figure 2). Analyses of the Pro12Ala distribution in the collective of patients with fatty liver disease (n = 363) revealed an increased incidence of the alanine mutant (3%) compared to the healthy population (1.5%). However, the difference was not statistically significant.

In order to identify the association of enhanced alanine occurrence, clinical data of patients with fatty liver disease were considered, revealing that 263 patients suffered from NAFLD, whereas fatty liver disease was linked to alcohol consumption in 100 cases (Table 3). In both cohorts, 12%-14% of the subjects were diabetes positive.

Furthermore, we analyzed the transaminase values in the two cohorts, showing a GPT/GOT ratio of 2 in

Table 3 Occurrence of diabetes mellitus in patients with fatty liver disease *n* (%)

	No C2 (NAFLD)	C2 (AFLD)
Diabetes mellitus positive	33 (12.5)	14 (14)
Diabetes mellitus negative	144 (54.8)	51 (51)
ND	86 (32.7)	35 (35)
Total	263 (100)	100 (100)

NAFLD: Non-alcoholic fatty liver disease; AFLD: Alcoholic fatty liver disease; ND: Not determined.

Table 4 Histological features in the fatty liver diseases cohort

Cases of fatty liver disease (*n* = 363)	*n* (%)
Steatosis	
S0	9 (2.5)
S1	169 (46.6)
S2	84 (23.1)
S3	101 (27.8)
Ballooning	
No ballooning	164 (45.2)
Stage 1	124 (34.2)
Stage 2	75 (20.7)
Inflammation	
G0	4 (1.1)
G1	217 (59.8)
G2	113 (31.1)
G3	29 (8)
Fibrosis	
F0	97 (26.7)
F1	116 (32)
F2	66 (18.2)
F3	56 (15.4)
F4	28 (7.7)

NAFLD patients, but a GPT/GOT ratio of 1 in sera of AFLD patients (Figure 3). The high GPT values in NAFLD patients are in accordance with numerous reports, characterizing the progress of steatohepatitis due to non-alcoholic steatosis in comparison to alcoholic steatosis[31]. With respect to the distribution of the Pro12 and the Ala12 alleles in these two cohorts, we found that in both the AFLD and the NAFLD collective the frequency of the alanine genotype was higher (about 3%) compared to the healthy population (Figure 2).

Association of the alanine allele with inflammation and fibrosis in fatty liver disease

We next addressed the question whether the elevated incidence of the alanine allele in the population of fatty liver diseases is associated with the grade of steatosis, ballooning, steatohepatitis, or liver fibrosis (Table 4).

The degree of steatosis is traditionally classified into mild (< 30%), moderate (30%-60%), and severe (> 60%). More than 50% of the patients with NAFLD and AFLD had developed severe steatosis, however, in patients carrying the alanine allele severe steatosis occurred slightly more often. Pronounced ballooning was observed likewise often in NAFLD patients with the mutant or the wild type isoform of the PPARγ2 (Table 5).

Histological scoring for inflammatory alterations and fibrosis according to the recommendations described by Kleiner *et al*[15] (Table 1) revealed that in most of the patients steatosis was accompanied by moderate, mild or severe steatohepatitis. In particular, in patients suffering from AFLD steatohepatitis has passed over to fibrosis in 71% of the cases.

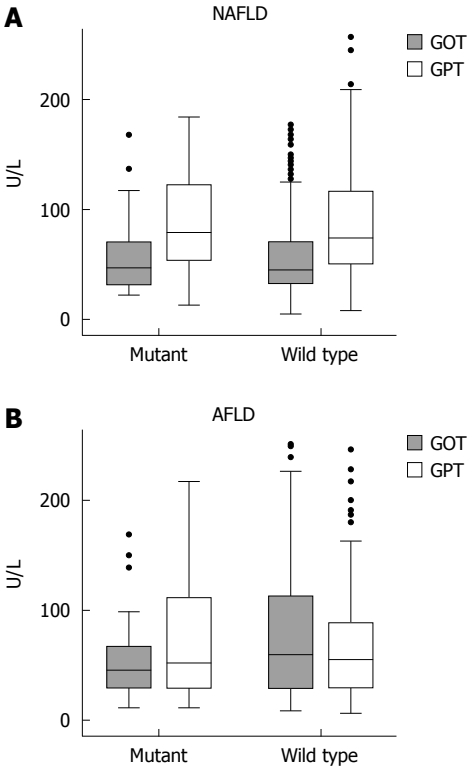

Figure 3 GOT and GPT levels in sera of non-alcoholic fatty liver disease (A) or alcoholic fatty liver disease (B) patients carrying the Ala mutated allele or the Pro wild type allele of the peroxisome-proliferator-activated receptor-γ2 gene. NAFLD: Non-alcoholic fatty liver disease; AFLD: Alcoholic fatty liver disease. Dots (●) indicate outliers that are not included between the whiskers.

Whereas in NAFLD patients inflammation was not significantly associated with the allelic incidence, in AFLD patients the frequency of the Ala12 variant of the PPARγ2 gene was significantly increased when prominent inflammation had occurred (P = 0.028). The higher risk of AFLD patients developing several inflammatory processes ending in liver fibrosis was also shown by elevated Odds ratios (Odd $_{\text{inflammation}}$ = 2.50, CI: 1.05-5.90 and Odd $_{\text{fibrosis}}$ = 2.48, CI: 0.81-7.53) (Table 6).

DISCUSSION

FLD has a high incidence of approximately 20% worldwide and is regarded as a major cause of liver-related morbidity and mortality due to its risk of progression into cirrhosis or hepatocellular carcinoma. Since the transcription factor PPARγ as been shown to be markedly involved in adipogenesis, hepatic lipid storage and metabolism, we first analyzed the frequency of the Pro12Ala polymorphism of the PPARγ gene in a German cohort of patients with FLD compared to German healthy blood donors. A highly sensitive and robust test was established which was certain to distinguish the Ala and the Pro alleles, even

Table 5 Occurrence of the wt and the mutated form of peroxisome proliferator-activated receptor-γ2 depending on the grade of steatosis and ballooning *n* (%)

	Steatosis						Ballooning					
	NAFLD			AFLD			NAFLD			AFLD		
	Total	Moderate (0-1)	Severe (2-3)	Total	Moderate (0-1)	Severe (2-3)	Total	-	+	Total	-	+
Allelic discrimination												
Mutation	66 (100)	27 (41)	39 (59)	26 (100)	12 (46)	14 (54)	66 (100)	28 (42)	38 (58)	26 (100)	19 (73)	7 (27)
Wild type	460 (100)	229 (50)	231 (50)	174 (100)	88 (51)	86 (49)	460 (100)	206 (45)	254 (55)	174 (100)	121 (70)	53 (30)
P value		0.112			0.417			0.411			0.454	
Odds ratio (CI)		1.43 (0.85-2.42)			1.19 (0.52-2.73)			1.10 (0.65-1.85)			0.84 (0.33-2.12)	

NAFLD: Non-alcoholic fatty liver disease; AFLD: Alcoholic fatty liver disease.

Table 6 Occurrence of the wt and mutated form of the peroxisome proliferator-activated receptor-γ2 depending on the grade of inflammation and fibrosis *n* (%)

	Inflammation						Fibrosis					
	NAFLD			AFLD			NAFLD			AFLD		
	Total	Moderate (0-1)	Severe (2-3)	Total	Moderate (0-1)	Severe (2-3)	Total	F0-F1	F2-F4	Total	F0-F1	F2-F4
Allelic discrimination												
Mutation	66 (100)	38 (58)	28 (42)	26 (100)	9 (35)	17 (65)	66 (100)	47 (71)	19 (29)	26 (100)	4 (15)	22 (85)
Wild type	460 (100)	296 (64)	164 (36)	174 (100)	99 (57)	75 (43)	460 (100)	327 (71)	133 (29)	174 (100)	54 (31)	120 (69)
P value		0.175			0.028			0.555			0.075	
Odds ratio (CI)		1.33 (0.79-2.25)			2.50 (1.05-5.90)			0.99 (0.56-1.76)			2.48 (0.81-7.53)	

NAFLD: Non-alcoholic fatty liver disease; AFLD: Alcoholic fatty liver disease.

though only low copy numbers from some FFPE biopsies might be available. The genotype distribution (77.5% wt, 21.2% heterozygous, and 1.5% homozygous Ala/Ala mutants) in the collective of the healthy blood donors resembles previous data collected on more than 600 Caucasians by Yen *et al*[32] and Ghoussaini *et al*[33]. This genotype distribution in Caucasians, however, differs from the Asian or African frequency, in which less Ala alleles of the PPARγ occur[23,32]. In contrast to the data of healthy blood donors, PCR analyses of DNA from subjects with FLD revealed an increased frequency of the homozygous Ala-subtype up to almost 3.5% in both the AFLD and the NAFLD collectives. Recent meta-analyses summarized data of the Pro12Ala polymorphism in patients with diabetes and identified the mutated Ala variant as a protection factor of diabetes type 2[34-36]. The malfunctioning Ala variant was also shown to be associated with coronary heart disease[37] and with obesity indicated by significant higher BMI in homozygous Ala carriers than in subjects expressing the heterozygous or the wild type PPARγ2 form[38,39]. Although in some reports higher insulin sensitivity and BMI could not be confirmed[40,41], a comprehensive study on 1170 British patients with coronary heart disease[42] and a meta-analysis including 19 136 subjects clearly identified the Ala carriers as individuals with significant higher BMI[43]. Additionally, cholesterol, LDL-cholesterol and apolipoprotein B concentrations are elevated in Ala carriers[42,44]. Therefore, this Ala-associated hyperlipidemia is assumed to be a reason for the 2-fold higher incidence of the Ala genotype in patients with FLD compared to healthy blood donors. Since free fatty acids are shown to be involved in upregulation of Fas/CD-95 death receptor[45], enhanced levels of circulating fatty acids due to impaired PPARγ function in Ala/Ala patients may result in apoptosis and inflammatory processes. In contrast to NAFLD, where no or only a moderate link of inflammatory progression to the Pro12Ala polymorphism was shown, a prominent risk of developing steatohepatitis was observed in AFLD patients carrying the Ala allele. This difference in the associated frequency of the Ala variant encoding the minor active PPARγ2 form[23] argues for a divergent role of the PPARγ2 in mechanisms of AFLD and NAFLD progression. The PPARγ2 isoform is upregulated by phosphatidylinositol 3-kinase activation in response to free fatty acids or by insulin[46]. In post-ischemic liver injury and also in alcohol-induced fibrosis, the PPARγ1 andγ2 variants were shown to be downregulated and to function protectively[47,48]. Therefore, the 2-3-fold higher risk of ALFD patients, but not of NAFLD patients, to develop inflammatory and fibrotic progression if they carry the malfunctioning Ala variant of PPARγ2, emphasizes a more prominent anti-inflammatory impact of the PPARγ2 in AFLD than in NAFLD wt-carriers.

The anti-inflammatory action of PPARγ was also demonstrated by previous studies on hepatic stellate cells[49,50], which take centre stage of sinusoidal liver fibrosis due to their tremendous matrix production and secretion of pro-inflammatory and pro-fibrotic mediators after myofibroblastic transition in chronic liver injury[16]. The authors show that PPARγs repressed in myofibroblastic hepatic

stellate cells. Additionally, the inflammatory chemokine expression by hepatic stellate cells is markedly inhibited in response to the activation of PPARγ by the agonistic ligand glitazone[49,50]. Taken into account that the malfunctioning Ala variant is associated with a higher risk of progression into steatohepatitis in AFLD patients these results lead to the suggestion that in particular inflammation and fibrosis of AFLD wt-patients can be attenuated by a treatment with PPARγ thiazolidinedione ligands such as rosiglitazone and pioglitazone. Patients with NASH, however, may benefit from glitazone therapy by other mechanisms like improved insulin sensitivity, decreased hyperlipidemia and impeded steatosis as a result of the therapeutic approach[51,52].

In conclusion, our data of a comprehensive study of the Pro12Ala polymorphism on biopsies with FLD, well classified concerning inflammatory and fibrotic alterations, revealed for the first time an association of the Pro12Ala polymorphism with the risk of developing ASH and suggests a more prominent anti-inflammatory influence of the PPARγ2 on progression of human AFLD than on NAFLD. Therefore, the Pro12Ala polymorphism should be studied on an expanded cohort of AFLD patients, in order to be later integrated in a panel of genetic markers applied for future improved prognosis of disease progression.

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COMMENTS

Background

Fatty liver diseases are becoming a common cause of chronic liver diseases in the Western countries encountered in about 20% of the general adult and child population. The spectrum of fatty liver diseases (FLD) independently of causative agents ranges from simple steatosis to steatohepatitis, which can progress to liver fibrosis ending up in cirrhosis or hepatocellular carcinoma. Members of the peroxisome proliferator-activated receptors (PPAR) seem to play a key role in the pathophysiology of FLD by modulating increased glucose uptake and hepatic triglyceride accumulation, but also perform anti-inflammatory signals when steatohepatitis has occurred. Since the transcription factor PPARγ has been shown to be markedly involved in adipogenesis, hepatic lipid storage and metabolism, we analyzed the frequency of the Pro12Ala polymorphism of the PPARγ gene in a German cohort of patients with FLD compared to German healthy blood donors.

Research frontiers

Due to its high impact as an insulin-sensitising transcription factor involved in adipogenesis and lipogenesis, the occurrence of single nucleotide polymorphisms (SNP) in the PPARγ gene was recently addressed by numerous reports studying subjects with insulin resistance, type 2 diabetes, arteriosclerosis, and hypertension. A prevalent SNP association with impaired lipid homeostasis was observed in terms of the N-terminal proline alanine exchange (Pro12Ala) of the extra domain in the PPARγ2 variant. The authors analyzed the frequency of the Pro12Ala polymorphism in the PPARγ gene by a highly sensitive LNA-probe based polymerase chain reaction approach in a total of 622 subjects of a Caucasian population, suffering from fatty liver disease (n = 359) or healthy blood donors (n = 263). In agreement with reports showing a high Ala allele prevalence in patients with impaired lipid metabolism in obese and adipose patient, the Ala allele also occurs more often in FLD patients than in the healthy control group. Interestingly, the interpretation and linkage of the allele frequency to histological evaluation and clinical data demonstrates a prominent risk in alcoholic fatty liver disease (AFLD) patients bearing the Ala allele to develop severe steatohepatitis and fibrosis.

Innovations and breakthroughs

In order to detect the Ala12Pro polymorphism in patients with fatty liver disease the authors established an assay using locked nucleotide acid probes for allelic discrimination. The authors' data of a comprehensive study of the Pro12Ala polymorphism on biopsies with FLD, well classified concerning inflammatory and fibrotic alterations, revealed in patients with AFLD, but not with non-alcoholic fatty liver disease (NAFLD), an significant association of the Pro12Ala polymorphism with the risk to develop steatohepatitits. Therefore, PPARγ is suggested to exert a higher anti-inflammatory impact in progression of human AFLD than NAFLD.

Applications

The association of the Pro12Ala polymorphism with the risk of developing inflammatory progression in patients with AFLD suggests a more prominent influence of PPARγ2 on progression of human AFLD than on NAFLD. Therefore, the Pro12Ala polymorphism should be studied on an expanded cohort of AFLD patients, in order to be later integrated in a panel of genetic markers applied for future improved prognosis of disease progression and therapy planning.

Peer review

This review article provides an overview of SLE-related gastrointestinal system involvements, and there are only few review article in the international literatures in recent years.

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BRIEF ARTICLE

Translation and validation of the Greek chronic liver disease questionnaire

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Abstract

AIM: To translate into Greek and validate the chronic liver disease questionnaire (CLDQ).

METHODS: Two hundred and six consecutive adult patients with the diagnosis of a chronic liver disease from 2 general hospitals in Athens were enrolled in the study from May to September 2008. In order to assess their quality of life (QOL) the CLDQ was applied. The instrument was translated from English, back translated and reviewed in focus groups within the framework of a large multicenter study. The measurements that were performed included: 2 independent sample *t* tests, one-way analysis of variance, reliability coefficients, explanatory factor analysis using a varimax rotation and the principal components method.

RESULTS: One hundred and twenty five (61%) patients were men, half were aged 40-59 years and > 33% were > 60 years old. Among the patients, 48 (23%) were hospitalized and 97 (47%) were cirrhotic according to the Child-Pugh score. The internal consistency of the Greek CLDQ version using Cronbach's alpha coefficient was found to be 0.93. Exploratory factor analysis identified 7 domains accounting for 65% of the variance of CLDQ items and only partially overlapping with those found in the original version. The area under the receiver operating characteristics curve was calculated at 0.813 and the logistic estimate for the threshold score of 167.50 provided a sensitivity of 74.3% and a specificity of 71.6% for the model.

CONCLUSION: Our data confirmed the validity of the Greek version of the CLDQ in identifying the QOL among patients with chronic liver disease.

Key words: Chronic disease; Questionnaires; Validation; Quality of life; Liver cirrhosis

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INTRODUCTION

Chronic liver disease encompasses a wide range of illnesses characterized by liver inflammation and progression to cirrhosis. Quality of life (QOL) is a concept that incorporates many aspects of an individual's experience, general well being and satisfaction, as well as social and physical functioning[1]. Health-related QOL is important when measuring the impact or burden of a chronic disease, such as liver disease, and is highly correlated with fatigue, loss of esteem, depression and disease complications[2]. In the last few decades, the assessment of health-related QOL has become an important outcome measure in clinical research in both gastroenterology and hepatology[3]. Whilst a variety of generic QOL measures have been developed, there is a need to develop specific instruments endowed with sufficient sensitivity to document clinically significant changes over time[4].

The chronic liver disease questionnaire (CLDQ) is a specific health-related QOL instrument designed for patients with liver disease, regardless of the underlying etiology and degree of disease. Its original version was developed first by Younossi *et al*[5] and has demonstrated appropriate validity and reliability. The CLDQ has already been cross-culturally adapted and validated into different languages in recently published studies[6-10]. Consequently, the aim of this paper was to: (1) report on the development of the Greek version of the CLDQ and on the validation procedures carried out; (2) examine the factorial structure of the Greek CLDQ; and (3) evaluate the sensitivity of the Greek CLDQ in assessing QOL over a range of cut-off scores among liver disease patients.

MATERIALS AND METHODS

CLDQ

The CLDQ is a 29-item self-reported scale consisting of statements describing QOL, and is divided into 6 domains including abdominal symptoms, fatigue, systemic symptoms, activity, emotional function and concern. All items refer to the previous 2 wk on a 7-point Likert scale, with 1 corresponding to the maximum frequency labeled as "all of the time" and 7 to the minimum labeled as "none of the time". Permission to reproduce and validate the CLDQ was provided by Younossi *et al*[5].

Greek version of CLDQ - translation and pilot study

The 29 items of the CLDQs were translated by 2 independent bilingual translators. Another native English speaker who did not have knowledge of the original instrument then back translated the Greek version. The backward translation was sent to a group of English experts for comments. The translated questionnaire was culturally adapted through a cognitive debriefing process that was used to identify any language problems and to assess the degree of respondents understanding of the item' s content that was meant to be elicited[5].

During this stage the reconciled Greek version of the CLDQ was pilot tested among 10 patients. As part of the cultural adaptation process, in-depth interviews were implemented with regard to the respondents understanding of the questionnaire with the purpose of revealing inappropriately interpreted items and translation alternatives. The participants gave their impression on the clarity of each item, the relevance of the content to their situation, the comprehensiveness of the instructions and their ability to complete it on their own. They were also encouraged to make suggestions whenever necessary. Finally, written comments made by the participants were included in the final Greek version of CLDQ.

Sample and data collection

Consecutive adult patients diagnosed with chronic liver disease, confirmed by laboratory tests, imaging studies and in most cases by liver histology, were asked to participate in our study. Enrolment started in May and ended in September 2008 among patients of the Gastroenterology Clinics of 2 general hospitals in Athens. Inclusion criteria were fluency in the spoken and written Greek language, age > 18 years and the existence of liver disease symptoms during the previous 3 mo. Non-Greek-speaking patients, patients who had undergone liver transplantation, patients with dementia or psychosis, and patients with refractory encephalopathy (grade Ⅱ and more) were excluded. To asses the severity of liver disease, the patients' Child-Pugh scores were calculated and patients' were classified as cirrhotic or not[11]. In total, 220 patients were approached and 206 patients agreed to participate (rate of attendance 93.6%). CLDQs were also completed by healthy participants (n = 208, controls) in order to perform receiver operating characteristics (ROC) analysis. The control group was selected randomly from a list of the Athens county population and was matched with cases by gender, age and educational background. One control was selected for one case participant. Both patients and healthy participants completed the CLDQ in the presence of a nurse.

All participants entering the study provided written informed consent after receiving a complete description of the study and having the opportunity to ask for clarifications. Along with the questionnaires there was a cover letter explaining the purpose of the study, providing the researchers' affiliation and contact information, and clearly stating that answers would be confidential and anonymity would be guaranteed in the final data reports.

Statistical analysis

Descriptive characteristics were determined for the sociodemographic variables of the sample and Student t tests were performed on the descriptive characteristics of the study population and the CLDQ score. All P-values were based on 2-sided tests and significance was defined as $P < 0.05$. The assumptions of normality, homogeneity and independence of the sample were checked. Reliability coefficients as measured by Cronbach's alpha were calculated for the CLDQ in order to assess reproducibility and consistency of the instrument. The underlying dimensions

Table 1 Patients' demographic and medical characteristics

	n (%)	Mean score	*P*-value
Sex			
Men	125 (60.7)	4.77 (1.26)	0.61
Women	81 (39.3)	4.85 (1.13)	
Age (yr)			
≤ 39	40 (19.4)	5.19 (1.15)	0.02
40-59	99 (48.1)	4.92 (1.22)	
≥ 60	67 (32.5)	4.40 (1.13)	
Educational level			
Primary	74 (35.9)	4.55 (1.19)	0.03
Secondary	85 (41.3)	4.84 (1.24)	
Higher	47 (22.8)	5.13 (1.09)	
Family status			
Married	144 (69.9)	4.74 (1.22)	0.27
Non-married	62 (30.1)	4.94 (1.17)	
Child-Pugh classification			
Class A	49 (23.8)	4.71 (1.06)	< 0.001
Class B	32 (15.5)	3.82 (1.12)	
Class C	16 (7.8)	3.36 (0.88)	
No Cirrhosis	109 (52.9)	5.34 (0.95)	
Etiology of liver disease			
Hepatitis B	78 (37.9)	5.07 (1.01)	< 0.001
Hepatitis C	65 (31.6)	4.97 (1.22)	
Autoimmune hepatitis	12 (5.8)	4.72 (1.31)	
Cirrhosis	36 (17.5)	3.92 (1.22)	
Other	15 (7.3)	4.87 (1.10)	

of the scale were checked with an explanatory factor analysis using a varimax rotation and principal components method as a descriptive method for analyzing grouped data[12]. Factor analysis using principal component analysis with varimax rotation was carried out to determine the dimensional structure of CLDQ using the following criteria: (1) eigenvalue > 1[13]; (2) variables should have a load > 0.50 on only one factor and < 0.40 on more factors; (3) the interpretation of the factor structure should be meaningful; and (4) the screen plot is accurate in the case where the means of communalities are above 0.60[14]. Computations were based on a covariance matrix, as all variables were receiving values from the same measurement scale[15]; Bartlett's test of sphericity with $P < 0.05$ and a Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy of 0.6 were used when performing this factor analysis. A factor was considered as important if its eigenvalue exceeded 1.0[13]. As the factor analysis found 2 independent domains, subsequent Cronbach's alpha measurements were separately performed for each domain, highlighting how the items were grouped together.

Sensitivity and specificity

The sensitivity and specificity were calculated at several cut-off scores of the CLDQ. A ROC analysis was carried out; this method allows the display of all the pairs of sensitivity and specificity values achievable as the threshold is changed from low to high scores, plotting the true-positive rate (sensitivity) on the vertical axis and the false-positive rate (1-specificity) on the horizontal axis. The area under the ROC curve is a quantitative indicator of the information content of a test and it may be interpreted as an estimate of the probability that a liver disease patient at random will, at each threshold, have a lower test score than a healthy participant.

Table 2 The 29 Greek chronic liver disease questionnaire items

	CLDQ item	mean ± SD
Q1	Abdominal bloating	5.08 ± 2.01
Q2	Tiredness or fatigue	3.98 ± 2.14
Q3	Bodily pain	5.22 ± 2.03
Q4	Sleepiness during the day	4.96 ± 1.89
Q5	Abdominal pain	5.67 ± 1.84
Q6	Shortness of breath	5.42 ± 2.02
Q7	Not eating enough	4.78 ± 2.35
Q8	Decreased strength	4.50 ± 2.21
Q9	Trouble in carrying or lifting heavy objects	4.59 ± 2.49
Q10	Anxiety	3.40 ± 2.29
Q11	Decreased energy	4.50 ± 2.25
Q12	Unhappiness	4.67 ± 2.02
Q13	Drowsiness	5.04 ± 1.87
Q14	Bothered by a limitation of the diet	3.17 ± 2.50
Q15	Irritability	4.33 ± 2.12
Q16	Difficulty in sleeping at night	4.91 ± 2.15
Q17	Abdominal discomfort	5.16 ± 2.05
Q18	Worries about the impact of the liver disease	3.95 ± 2.22
Q19	Mood swings	4.55 ± 2.01
Q20	Difficulty falling asleep at night	5.07 ± 2.15
Q21	Muscle cramps	5.50 ± 1.87
Q22	Worries that symptoms will develop into major problems	4.21 ± 2.05
Q23	Dry mouth	5.17 ± 2.06
Q24	Depression	4.74 ± 2.04
Q25	Worries that the condition is getting worse	4.28 ± 2.12
Q26	Problems	5.75 ± 1.74
Q27	Itching	5.78 ± 1.91
Q28	Worries about never feeling any better	4.46 ± 2.22
Q29	Concerned about the availability of a liver in the case of a transplant	6.41 ± 1.42

CLDQ: Chronic liver disease questionnaire.

RESULTS

Patients' demographic and medical characteristics are shown in Table 1. Almost 61% of the sample were men and nearly half of the sample ($n = 99$, 48%) were aged 40-59 years with 33% ($n = 67$) aged ≥ 60 years old. Seventy four (36%) had a primary level of education, 41% ($n = 85$) a secondary level and 23% ($n = 47$) had higher education. The majority of the patients (70%) were married. Regarding the patients' disease status, 23% were hospitalized while 47% were classified as having severe cirrhotic liver disease based on the Child-Pugh classification. The most common etiology for their liver disease was viral hepatitis (69.5%) and cirrhosis (18%). Table 1 also depicts the differences in the mean total score of CLDQ according to the patients' characteristics. More specifically, older age, lower educational level and cirrhotic disease were found to be associated with lower CLDQ scores and therefore with lower QOL among patients.

The mean score of the CLDQ was 4.81 [standard deviation (SD) 2.01] and ranged from 3.17 to 6.41 (Table 2). The communalities for the Greek CLDQs are presented in Table 3. The internal consistency characteristics of the

Table 3 Inter-item correlation matrix for Greek chronic liver disease questionnaire

	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11	Q12	Q13	Q14	Q15	Q16	Q17	Q18	Q19	Q20	Q21	Q22	Q23	Q24	Q25	Q26	Q27	Q28	Q29
Q1	1.00	0.45	0.42	0.22	0.45	0.37	0.29	0.35	0.34	0.30	0.41	0.20	0.18	0.21	0.21	0.31	0.59	0.26	0.23	0.36	0.16	0.22	0.28	0.20	0.29	0.25	0.36	0.31	0.16
Q2	0.45	1.00	0.56	0.34	0.42	0.57	0.52	0.76	0.67	0.30	0.75	0.34	0.42	0.22	0.30	0.43	0.43	0.40	0.44	0.41	0.21	0.35	0.33	0.31	0.38	0.30	0.32	0.39	0.13
Q3	0.42	0.56	1.00	0.39	0.37	0.33	0.28	0.50	0.41	0.29	0.52	0.37	0.42	0.12	0.18	0.32	0.39	0.24	0.38	0.34	0.21	0.12	0.37	0.30	0.18	0.20	0.24	0.18	0.10
Q4	0.22	0.34	0.39	1.00	0.28	0.34	0.29	0.33	0.30	0.26	0.40	0.24	0.72	0.17	0.16	0.32	0.22	0.20	0.33	0.28	0.28	0.23	0.29	0.19	0.26	0.30	0.14	0.26	0.31
Q5	0.45	0.42	0.37	0.28	1.00	0.41	0.32	0.40	0.36	0.18	0.43	0.18	0.26	0.24	0.17	0.35	0.50	0.34	0.24	0.28	0.14	0.20	0.27	0.17	0.26	0.17	0.23	0.22	0.22
Q6	0.36	0.57	0.33	0.34	0.41	1.00	0.49	0.58	0.57	0.24	0.61	0.20	0.32	0.35	0.28	0.48	0.47	0.39	0.33	0.47	0.29	0.28	0.32	0.29	0.29	0.21	0.32	0.35	0.20
Q7	0.29	0.52	0.28	0.29	0.32	0.49	1.00	0.54	0.50	0.36	0.60	0.38	0.28	0.28	0.34	0.40	0.39	0.41	0.40	0.39	0.31	0.36	0.19	0.36	0.39	0.26	0.27	0.39	0.12
Q8	0.35	0.76	0.50	0.33	0.40	0.58	0.54	1.00	0.78	0.31	0.87	0.44	0.39	0.28	0.26	0.41	0.43	0.42	0.44	0.42	0.20	0.35	0.28	0.38	0.42	0.32	0.32	0.40	0.13
Q9	0.34	0.67	0.41	0.30	0.36	0.57	0.50	0.78	1.00	0.28	0.77	0.32	0.35	0.32	0.18	0.39	0.48	0.30	0.37	0.38	0.19	0.24	0.32	0.21	0.31	0.23	0.36	0.32	0.09
Q10	0.30	0.30	0.29	0.26	0.18	0.24	0.36	0.31	0.28	1.00	0.37	0.41	0.29	0.16	0.45	0.30	0.23	0.36	0.48	0.32	0.18	0.27	0.24	0.36	0.35	0.29	0.08	0.29	0.09
Q11	0.41	0.75	0.52	0.40	0.43	0.61	0.60	0.87	0.77	0.37	1.00	0.39	0.40	0.31	0.31	0.42	0.43	0.42	0.47	0.43	0.26	0.38	0.29	0.36	0.42	0.33	0.32	0.40	0.13
Q12	0.20	0.34	0.37	0.24	0.18	0.20	0.38	0.44	0.32	0.41	0.39	1.00	0.30	0.23	0.35	0.36	0.29	0.34	0.63	0.36	0.08	0.35	0.24	0.68	0.44	0.22	0.16	0.41	0.10
Q13	0.18	0.42	0.42	0.72	0.26	0.32	0.28	0.39	0.35	0.30	0.40	0.30	1.00	0.10	0.15	0.30	0.28	0.23	0.36	0.34	0.28	0.15	0.32	0.26	0.25	0.40	0.20	0.25	0.18
Q14	0.21	0.22	0.12	0.17	0.24	0.35	0.28	0.28	0.32	0.16	0.31	0.23	0.10	1.00	0.14	0.22	0.29	0.36	0.12	0.28	0.06	0.20	0.22	0.14	0.23	0.07	0.17	0.27	0.17
Q15	0.21	0.30	0.18	0.16	0.17	0.28	0.34	0.29	0.18	0.45	0.31	0.35	0.15	0.14	1.00	0.34	0.20	0.46	0.43	0.35	0.13	0.33	0.04	0.32	0.37	0.21	0.12	0.33	0.13
Q16	0.31	0.43	0.32	0.32	0.35	0.48	0.40	0.41	0.39	0.30	0.42	0.36	0.30	0.22	0.34	1.00	0.36	0.30	0.40	0.84	0.28	0.17	0.32	0.38	0.20	0.25	0.22	0.24	0.24
Q17	0.59	0.43	0.39	0.22	0.50	0.47	0.39	0.43	0.48	0.23	0.43	0.29	0.28	0.29	0.20	0.36	1.00	0.36	0.31	0.38	0.22	0.28	0.39	0.29	0.36	0.19	0.37	0.35	0.12
Q18	0.26	0.40	0.24	0.20	0.34	0.39	0.41	0.42	0.30	0.34	0.42	0.34	0.23	0.36	0.46	0.30	0.36	1.00	0.40	0.33	0.15	0.51	0.11	0.38	0.53	0.13	0.19	0.53	0.20
Q19	0.23	0.44	0.38	0.33	0.24	0.33	0.40	0.44	0.37	0.48	0.47	0.63	0.36	0.12	0.43	0.40	0.31	0.40	1.00	0.42	0.14	0.36	0.27	0.60	0.45	0.20	0.24	0.45	0.15
Q20	0.36	0.41	0.34	0.28	0.28	0.47	0.39	0.42	0.38	0.32	0.43	0.36	0.34	0.28	0.35	0.84	0.38	0.33	0.42	1.00	0.30	0.22	0.34	0.39	0.22	0.29	0.26	0.27	0.21
Q21	0.16	0.21	0.21	0.28	0.14	0.29	0.31	0.20	0.19	0.18	0.26	0.08	0.28	0.06	0.13	0.28	0.22	0.15	0.14	0.30	1.00	0.14	0.25	0.21	0.15	0.15	0.23	0.13	0.18
Q22	0.22	0.35	0.12	0.23	0.20	0.28	0.36	0.35	0.24	0.27	0.38	0.35	0.15	0.20	0.33	0.17	0.28	0.51	0.36	0.22	0.14	1.00	0.16	0.43	0.81	0.15	0.10	0.74	0.15
Q23	0.27	0.33	0.37	0.29	0.27	0.32	0.19	0.28	0.32	0.24	0.29	0.24	0.32	0.22	0.04	0.32	0.39	0.11	0.27	0.34	0.25	0.16	1.00	0.26	0.15	0.28	0.32	0.14	0.17
Q24	0.20	0.31	0.30	0.19	0.17	0.29	0.36	0.38	0.21	0.36	0.36	0.68	0.26	0.14	0.32	0.38	0.29	0.38	0.60	0.39	0.21	0.43	0.26	10.0	0.50	0.19	0.18	0.49	0.09
Q25	0.29	0.38	0.18	0.26	0.25	0.29	0.39	0.42	0.31	0.35	0.42	0.44	0.25	0.23	0.37	0.20	0.36	0.53	0.45	0.22	0.15	0.81	0.15	0.50	1.00	0.15	0.11	0.86	0.08
Q26	0.25	0.30	0.20	0.30	0.17	0.21	0.26	0.32	0.23	0.29	0.33	0.22	0.40	0.07	0.21	0.25	0.19	0.13	0.20	0.29	0.15	0.15	0.28	0.19	0.15	1.00	0.04	0.19	0.13
Q27	0.36	0.32	0.24	0.14	0.23	0.32	0.27	0.32	0.36	0.08	0.32	0.16	0.20	0.17	0.12	0.22	0.37	0.19	0.24	0.26	0.23	0.10	0.32	0.18	0.11	0.04	1.00	0.12	0.17
Q28	0.31	0.39	0.18	0.26	0.22	0.35	0.39	0.40	0.32	0.29	0.40	0.41	0.25	0.27	0.33	0.24	0.35	0.53	0.45	0.27	0.13	0.74	0.14	0.49	0.86	0.19	0.12	10.0	0.13
Q29	0.16	0.13	0.10	0.31	0.22	0.20	0.12	0.13	0.09	0.09	0.13	0.10	0.18	0.17	0.13	0.24	0.12	0.20	0.15	0.21	0.18	0.15	0.17	0.09	0.08	0.13	0.19	0.13	1.00

CLDQ: Chronic liver disease questionnaire.

Table 4 Exploratory factors and explained variance after rotation for the Greek chronic liver disease questionnaire

Factors		Rotation sums of squared loadings				
		Rescaled loadings	Eigen values	% of variance	Cumulative variance	Cronbach's alpha
Factor Ⅰ	Question 2	0.72				
	Question 8	0.84				
	Question 9	0.84	4.08	14.07	14.07	0.91
	Question 11	0.82				
	Question 18	0.55				
Factor Ⅱ	Question 22	0.85				
	Question 25	0.87	3.24	11.07	25.24	0.93
	Question 28	0.85				
Factor Ⅲ	Question 1	0.79				
	Question 5	0.57	2.78	9.57	34.81	0.80
	Question 17	0.74				
Factor Ⅳ	Question 4	0.83	2.49	8.6	43.41	0.91
	Question 13	0.8				
Factor Ⅴ	Question 12	0.79	2.42	8.35	51.76	0.79
	Question 24	0.73				
Factor Ⅵ	Question 16	0.79	2.11	7.27	59.03	0.84
	Question 20	0.77				
Factor Ⅶ	Question 10	0.72	1.68	5.78	64.81	0.67
	Question 15	0.73				

Greek CLDQ showed good reliability as the Cronbach's alpha was 0.93 for the total scale (Items 1-29). The exploratory factor analysis on the 29 items of the CLDQ revealed 2 orthogonal d (KMO measure of sampling adequacy = 0.886 and Bartlett's test of sphericity = 3422.25, df = 406, $P < 0.001$). Factor analysis indicated there are 7 principal domains in the model which explained 64.81% as presented in Table 4. The first domain (F1) included the following items: 2 (fatigue), 8 (decreased strength), 9 (trouble lifting heavy objects) and 11 (decreased level of energy) and this domain was named as "Fatigue". The second domain (F2) was composed of items 18 (concern about the impact of liver disease on the family), 22 (concern that symptoms will develop into a major symptom), 25 (concern about the condition getting worse) and 28 (concern about never feeling any better). Therefore F2 represented "Concern". The third factor (F3) included the following items: 1 (abdominal bloating), 5 (abdominal pain) and 17 (abdominal discomfort) and was named as "Abdominal symptoms". The fourth domain (F4) included the following items: 4 (feeling sleepy during the day) and 13 (drowsiness) and we named this as "Activity". The fifth factor (F5) was composed of items 12 (unhappiness) and 24 (feeling depressed). Therefore F5 represents "Emotional function". The sixth domain (F6) was composed of items 20 (unable to fall asleep at night) and 16 (difficulty sleeping at night). Therefore F6 represents "Sleeping disorders". The seventh domain (F7) was composed of items 10 (anxiety) and 15 (irritability). Therefore F7 represents "Anxiety". Cronbach's alpha for the 7 domains ranged from 0.67 (F7) to 0.93 (F2).

The Greek version of the CLDQ was well accepted by the patients. It was easily and very quickly (approximately 10 min) completed. The questions appeared to be relevant, reasonable, unambiguous and clear. Therefore, face validity was considered to be very good. The overall accuracy of the Greek CLDQ, as an instrument for assessing QOL among liver disease patients can be described as the area under its ROC curve calculated as 0.813 (SD = 0.021, Asymp. Sig < 0.0001). Table 5 presents the sensitivity and specificity values for the different cut-off values of the ROC analysis. A 167.50 cut-off score of the CLDQ provided the best sensitivity (74.3%) and specificity (71.6%). Figure 1 depicts the accuracy of the Greek CLDQ for assessing the level of QOL among patients with liver disease.

Table 5 Sensitivity and specificity values of different cut-off scores of the Greek chronic liver disease questionnaire for identifying level of quality of life

Threshold scores	Sensitivity (%)	Specificity (%)
147.50	57.8	99.0
157.50	64.6	82.2
167.50	74.3	71.6
175.50	85.4	55.8
185.50	92.2	37.0
194.50	98.5	16.8

DISCUSSION

CLDQ is a non-generic, disease-specific instrument for assessing QOL among liver disease patients. Our validation study provided a Cronbach's alpha equal to 0.93 and the factor analysis identified 7 domains with Cronbach's alpha ranging from 0.67 to 0.93 and included "Fatigue", "Concern", "Abdominal symptoms", "Emotional function", "Sleeping disorders", "Anxiety" and "Activity". Those domains explained 65% of the total variance. The ROC analysis presented the highest sensitivity and specificity at the overall score of 167.50, which can be considered as the cut-off score under which QOL can be assessed accurately.

CLDQ has already been validated in many countries

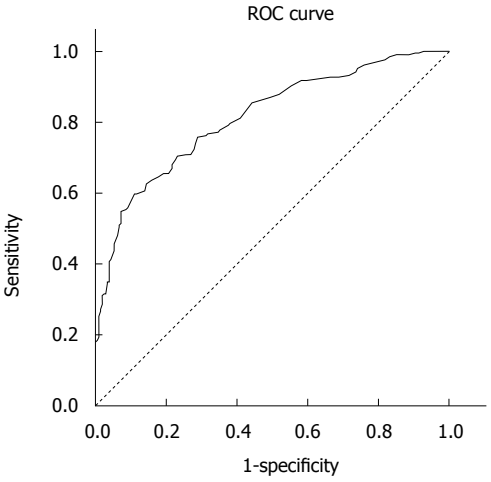

Figure 1 Receiver operating characteristics curve for the Greek chronic liver disease questionnaire. Diagonal segments are produced by ties. ROC: Receiver operating characteristics.

including Spain, Italy, Germany, Lithuania and Thailand and has shown remarkable reproducibility and comparability[6-10]. The overall Cronbach's alpha for the Greek CLDQ was found to be the same as those reported by the Spanish, Lithuanian and Thailand validation teams whereas the German validation study reported a Cronbach's alpha ranging from 0.69 to 0.95 among different domains. The cumulative variance of the Greek validated questionnaire is similar to the Spanish (68%) and Italian (65.4%) versions.

In comparing the structure of the Greek CLDQ with those of other countries we identified both discrepancies and similarities. The factor analysis of the Greek questionnaire revealed significance in 18 out of the 29 items included in the original one. The Greek validated version of CLDQ revealed a 7 domain structure similar to the Spanish validated questionnaire as opposed to the 6 domains of the original U.S. version[5] and the Italian version[10]. Additionally, the Greek version did not include questions regarding systemic symptoms which are included in the original version and in the existing validated versions of the questionnaire (Italian and Spanish)[9,10]. Sleepiness and drowsiness constitute a new domain in the Greek validation which was named "Activity" where as in both the Spanish[9] and in the original validation[5] they are included under the domain "Fatigue" and in the Italian version[10] under the domain "Somatic symptoms". "Anxiety" (including anxiety and irritability items) also consist of a separate domain in our validation whereas in the other validation studies these items are included in the domain of "Emotional symptoms". The "Fatigue" domain included the definitions of "felt drowsy" and "felt sleepy during the day" in the original and Spanish version[9] whereas in the Greek version these items are included in a new domain which was named as "Sleeping disorders". Only the factor comprising items that explored patient concern is common among the different versions.

The ROC analysis confirmed the effectiveness of CLDQ in assessing health-related QOL in the range of cut-off scores proposed. In our study, the high sensitivity (74.3%) of the 167.50 score allows the use of this cut-off score in the clinical assessment of QOL. If a health professional would like to use the Greek CLDQ for QOL assessment then these different cut-offs should be used. It is very important for the CLDQ to be used as a diagnostic tool in clinical practice which may allow health care professionals to understand the impact of health care interventions on the patient's everyday life, rather than the effects of treatment on their bodies[16]. Additional, the Greek CLDQ's use provides a basis for the holistic view of the patient and therefore may help facilitate a dialogue with patients with low QOL.

In general, this is the first study to validate the Greek CLDQ which is recommended to be incorporated into research and clinical practice to allow international comparison of the results of separate national studies[4]. An important strength of our study is that this is the first study to perform a ROC analysis which provided us with a cut-off score for assessing QOL accurately among patients with liver disease. Our findings also revealed a different structure of the questionnaire after the factor analysis which underlines the necessity of cultural validation and adaptation of the questionnaire before its use in specific countries. A limitation of this validation study was that there was no test-retest, because it may have resulted in a low correlation due to an actual change in the QOL symptoms. Additionally, the high percentage of patients with a cirrhotic liver disease might have affected the CLDQ score as the complications associated with cirrhosis, such as hepatic encephalopathy, have been shown to negatively influence physical and mental domains of QOL[17]. On the other hand, the exclusion of the patients with hepatic encephalopathy of grade Ⅱ or more might have reduced this error. In addition, the ethnic and cultural background of the patients may have had an effect on the score of CLDQ whereas previous studies have reported no differences in the CLDQ score among different ethnic groups[8,17].

The Greek version of the CLDQ has shown a satisfactory reliability and the factor analysis indicated 7 factors that were of interest. We can therefore assert that it is a reliable and valid tool for identifying QOL among liver disease patients and it can be used by health professionals in their clinical practice to improve assessment of patients with low scores. Our findings, however, need to be confirmed by future cross-sectional and cohort studies.

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COMMENTS

Background

Chronic liver disease questionnaire (CLDQ) is a disease specific instrument for assessing quality of life (QOL) among liver disease patients. In the last few decades, the assessment of QOL related to chronic liver disease has become an important outcome measure in clinical research.

Research frontiers

The CLDQ has already been cross-culturally adapted and validated into different languages. In the current study, the authors aimed to translate and validate the Greek version of the CLDQ.

Innovations and breakthroughs

The authors' findings revealed a different structure of the questionnaire after the factor analysis, which underlines the necessity of cultural validation and adaptation of the questionnaire before its use in specific countries. This is the first study to validate the Greek CLDQ which therefore should be incorporated into research and clinical practice so as to allow international comparison of the results of separate national studies. An important strength of the study is that this is the first study to perform a receiver operating characteristics (ROC) analysis which provided us with a cut-off score for accurately assessing QOL among patients with liver disease.

Applications

The Greek version of the CLDQ has shown a satisfactory reliability. The authors can therefore assert that it is a reliable and valid tool for identifying QOL among liver disease patients and it can be used by health professionals in their clinical practice to improve assessment of patients with low scores.

Terminology

ROC curve: a graphical plot of the sensitivity, or true positives, *vs* (1-specificity), or false positives, for a binary classification system as its discrimination threshold is varied.

Peer review

Dr. Zoi Kollia and colleagues validated the Greek version of the CLDQ to assess QOL factors for patients with chronic liver diseases. The establishment of QOL measures or indices is very important to assess what physical or mental status a patient is in and to compare multinational patients. This study provides an important clue to assess QOL of patients with chronic liver diseases.

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BRIEF ARTICLE

Serum immune-activation potency and response to anti-TNF-α therapy in Crohn's disease

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Abstract

AIM: To study whether immune-activation stage in serum of adult Crohn's disease (CD) patients correlates with disease activity and with treatment response to anti-tumor necrosis factor-α (TNF-α) therapy.

METHODS: Serum samples were obtained from 15 adult CD patients introduced to anti-TNF-α therapy. The individual stage of immune activation was studied applying our new *in vitro* assay, in which target cells (donor derived peripheral blood mononuclear cells) were cultured with patient serum and the T-cell activation induced by the patient serum was studied using a panel of markers for effector [interferon γ (IFNγ), interleukin (IL)-5] and regulatory T-cells [forkhead transcription factor 3 (FOXP3) and glucocorticoid-induced tumour necrosis factor receptor (GITR)]. The endoscopic disease activity was assessed with the Crohn's disease endoscopic index of severity (CDEIS) before and 3 mo after therapy with an anti-TNF-α agent.

RESULTS: Low induction of FOXP3 and GITR in target cells cultured in the presence of patient serum was associated with high disease activity i.e. CDEIS assessed before therapy (r = -0.621, P = 0.013 and r = -0.625, P = 0.013, respectively). FOXP3 expression correlated inversely with pre-treatment erythrocyte sedimentation rate (r = -0.548, P = 0.034). Low serum induced FOXP3 (r = -0.600, P = 0.018) and GITR (r = -0.589, P = 0.021) expression and low IFNγ secretion from target cells (r = -0.538, P = 0.039) associated with treatment response detected as a decrease in CDEIS.

CONCLUSION: The immune-activation potency in the patient serum prior to anti-TNF-α therapy reflected intestinal inflammation and the therapeutic response.

Key words: Crohn's disease endoscopic index of severity; Forkhead transcription factor 3; Glucocorticoid-induced tumour necrosis factor receptor; Infliximab; Inflammatory bowel disease

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INTRODUCTION

Tumor necrosis factor-α (TNF-α), a pro-inflammatory protein secreted mainly by monocytes, macrophages and T-cells, has a central role in the disease pathogenesis of Crohn's disease (CD)[1-3]. TNF-α is elevated in stools and mucosa of CD patients[4-6].

The chimeric TNF-α-antibody infliximab and human IgG_1 TNF-α-antibody adalimumab are indicated for the treatment of moderate to severe CD[7,8]. During treatment with an anti-TNF-α agent, clinical improvement is accompanied by significant healing of endoscopic lesions and the disappearance of mucosal inflammatory infiltrate[9]. The therapeutic response, however, is not uniform and a significant proportion of patients are non-responders. In the ACCENT I study, 58% of patients with moderate CD responded to the first infusion of infliximab and with adalimumab 24%-36% of TNF-α-antibody naïve patients responded to the induction of therapy[7,8].

There are a few studies exploring immunological markers that reflect treatment response to anti-TNF-α therapy in CD. Elevated concentration of mucosal nuclear NFκBp65 and high secretion of TNF-α by cells of peripheral blood cultivation precede clinical relapse[10]. In the study by Mäkitalo *et al*[11], the expression profile of the macrophage tissue inhibitor of metalloproteinase (TIMP)-1 and stromal TIMP-3 in the intestine correlated positively with the Crohn's disease endoscopic index of severity (CDEIS) and the down regulation of matrix metalloproteinase-9 (MMP9) production of macrophages correlated with histological improvement during anti-TNF-α therapy. Further, in another study serum MMP9 levels seemed to decrease particularly in those CD patients who responded to infliximab therapy[12].

Recently, we described a novel approach to study individual treatment responses at an early phase of glucocorticoid therapy. In that study we investigated pediatric patients with inflammatory bowel disease (IBD) introduced to systemic glucocorticoids and showed that patient serum modified the expression of T-cell signalling markers on target cells (peripheral blood mononuclear cells from a healthy donor)[13].

Encouraged by this finding we applied the same method to study the individual immune-activation potency in adult CD patients starting anti-TNF therapy. We measured the expression of the regulatory T-cell markers forkhead transcription factor 3 (FOXP3) and glucocorticoid-induced tumour necrosis factor receptor (GITR), and cytokines interferon γ (IFNγ), interleukin-5 (IL-5) and IL-17 induced in the target cells by patient serum in 15 adult CD patients at the beginning of anti-TNF-α therapy. We found that prior to anti-TNF-α therapy the ability of patient serum to modulate the FOXP3 and GITR activation of the target cells mirrored the disease activity and the individual therapeutic response in the gut assessed with the CDEIS.

MATERIALS AND METHODS

Subjects

Fifteen adult patients (6 females) with established CD were introduced to an anti-TNF-α agent due to an acute flare ($n = 6$), chronic active disease (6), or rapid postoperative reoccurrence of the disease (3; Table 1). Fourteen patients received infliximab infusion 5 mg/kg at week 0 and 8. One patient received an adalimumab induction dose 80 mg subcutaneously (*s.c.*) at week 0, followed by 40 mg *s.c.* every other week until week 8. After the beginning of the anti-TNF-α treatment, corticosteroids were tapered off.

Table 1 Patient characteristics

Age (yr): median (range)	25 (19-44)	
Disease duration, years median (range)	5.1 (0.4-27)	
	No. of patients	%
Disease location		
Ileum	2	13
Colon	4	27
Ileocolon	9	60
Disease type		
Inflammatory	7	47
Stricturing	5	33
Inflammatory + perianal	3	20
Prior anti-TNF-therapy	4	27
Prior bowel operation	4	27
Smokers	7	47
Baseline concomitant medication		
Azathioprine/6-mercaptopurine	10	67
Methotrexate	2	13
Corticosteroids	10	67
Mesalamine or sulphasalazine	13	87
Week 12 concomitant medication		
Azathioprine/6-mercaptopurine	13	87
Methotrexate	2	13
Corticosteroids	1	6.7
Mesalamine or sulphasalazine	11	73
CDAI at baseline, median (range)	158 (49-605)	
CDAI at week 12, median (range)	66 (24-202)	

CDAI: Crohn's disease activity index; TNF: Tumor necrosis factor.

All patients underwent an ileocolonoscopy before the introduction of anti-TNF-α therapy (median 7 d, range 1-38 d) and the endoscopic assessment of treatment response was performed at week 12 (week 10 for the adalimumab-treated patient). The endoscopic activity was graded according to the CDEIS[14,15]. This score is based on the presence of superficial or deep ulcerations, proportion of affected and ulcerated surface, and presence of either ulcerated or non-ulcerated stenosis in the terminal ileum and four segments of the colon (right, transverse, left colon and sigmoid, and rectum)[14]. Clinical disease activity was assessed with the Crohn's disease activity index (CDAI)[16].

Exclusion criteria were contraindication to anti-TNF-α treatment, pregnancy, history of extensive bowel resection, ostomy, long-term use of nonsteroidal anti-inflammatory drugs, or perianal fistulating disease without luminal inflammation.

Blood samples and fecal calprotectin

A serum sample for the target cell assay was provided at the time of the first ileocolonoscopy. The routine blood samples for serum C-reactive protein (CRP, normal value < 10 mg/L), erythrocyte sedimentation rate (ESR) and

fecal samples for measurement of calprotectin (PhiCal Test, Calpro AS, Oslo, Norway[17,18]) were obtained by the time of the endoscopies and 3 mo after the first anti-TNF-α dose[15].

Laboratory assay for systemic immunological effects

The assay for the assessment of individual stages of immunoactivation by applying patient serum in an in vitro culture of donor derived peripheral blood mononuclear cells (PBMC, target cells) is described recently in detail[13]. In brief, healthy donor (male 34 years) derived PBMC were separated by Ficoll-Paque (Amersham Biosciences) centrifugation (800 × G, 25 min) and cultured in the presence of the patients inactivated (35 min in 56℃) serum at an end concentration of 8%, either at resting state or activated with mitogen phytohemagglutin (PHA, 5 μg/mL). Serum of a healthy donor (male 27 years) was used as the methodological control between cell culture plates. After 72 h incubation at 37℃ in humified atmosphere with 5% CO2/air the supernatants were collected and stored at -70℃. This assay was performed with serum samples drawn prior to first anti-TNF-α infusion.

ELISA for IFNγ, IL-5 and IL-17

IFNγ, IL-5 and IL-17 were measured with ELISA in duplicate from the supernatants collected from the target cell cultures (see above) incubated with patient serum. IFNγ and IL-5 was detected as described before[19,20]. IL-17 was measured according to the manufacturer's protocol (Catalogue no: DY317; R&D Systems, United Kingdom). We subtracted the non-stimulated value from the stimulated value to obtain the Δ-value for statistical analyses.

Quantitative reverse transcriptase-polymerase chain reaction

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) process was recently described in detail[13]. Briefly, total RNA was isolated from cell samples with the GenElute Mammalian total RNA miniprep kit (Sigma-Aldrich), and the RNA concentration was measured by a spectrophotometer (ND-1000, NanoDrop Technologies Inc, Wilmington, DE, USA). Reverse transcription was performed by using TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA) with additional treatment of total RNA at 10 ng/μL with DNAse I (0.01 U/μL) (Roche Diagnostics, Mannheim, Germany) to eliminate genomic DNA. Quantitative RT-PCR was performed using predesigned FAM-labelled TaqMan Gene Expression Assay reagents (Applied Biosystems) and the ABI Prism 7700 Sequence Detection System (Applied Biosystems) in triplicate wells. Assay reagents for FOXP3 (Hs00203958_m1), GITR (Hs00188346_m1), IFNγ (Hs00174143_m1) and 18s RNA (Hs99999901_s1) were used. The difference value (ΔCt) is the normalised quantitative value of the expression level of the target gene achieved by subtracting the Ct value of the reference gene (18s) from the Ct value of the target gene. An exogenous cDNA pool calibrator was collected from PHA stimulated PBMC and considered as an interassay standard to which normalized samples were compared. ΔΔCt is the difference between the ΔCt of the analyzed sample and ΔCt of the calibrator. Calculation of $2^{-\Delta\Delta Ct}$ gives a relative amount of the target gene in analyzed sample compared with the calibrator, both normalized to an endogenous control (18S). For presentations the relative amount of target genes was multiplied by 1000 and expressed as relative units.

Statistical analysis

Results are reported as median. Comparison between two dependent samples was calculated with Wilcoxon rank test and two independent samples was calculated with Mann-Whitney *t*-test. The two-tailed Spearman's rho was used for calculation of the correlations and Kruskall-Wallis test served in exploring associations between groups (SPSS 16.0 program). $P < 0.05$ was set for statistical significance.

Ethics

All patients gave their informed written consent for participation in this study approved by the ethics committee of the Helsinki University Central Hospital.

RESULTS

Patient serum induced IFNγ, FOXP3 and GITR specific mRNA expression and secretion of IFNγ, IL-5 and IL-17 from target cells

The expression levels of IFNγ, FOXP3 and GITR specific mRNA in both resting and activated target cells cultured in the presence of CD patient serum obtained before anti-TNF-α therapy is shown in Table 2. Also, the secretion of IFNγ, IL-5 and IL-17 from activated target cells is shown in Table 2. The secretion of IFNγ, IL-5 and IL-17 from resting target cells was below detection limits.

The type of CD or localization was not associated with the level of IFNγ, FOXP3 and GITR specific mRNA expression or IFNγ, IL-5 and IL-17 secretion from target cells (all P = NS).

CDEIS

During anti-TNF-α therapy the CDEIS decreased from a median of 13 points (range 1.8-25) to 4.8 points (range 0-11, P = 0.002). 12/15 patients responded to therapy, while 3 patients had no decrease in the CDEIS.

Correlations between the target cell responses and pre-treatment the CDEIS

The expression of regulatory T-cell markers FOXP3 and GITR specific mRNA in activated target cells cultured with patient serum correlated inversely with the pre-treatment CDEIS (FOXP3 r = -0.621, P = 0.013 and GITR r = -0.625, P = 0.013; Figure 1). A trend towards an inverse correlation between IFNγ mRNA expression and the pre-treatment CDEIS was observed (r = -0.446, P = 0.095). There was no correlation between IFNγ, IL-5 or IL-17 secretion from target cells and the pre-treatment CDEIS (*P*

Table 2 The effect of Crohn's disease patient serum withdrawn before anti-tumor necrosis factor-α therapy on forkhead transcription factor 3, glucocorticoid-induced tumour necrosis factor receptor and interferon γ specific mRNA expression (relative units) and interferon γ, interleukin-5 and interleukin-17 secretion (pg/mL) from peripheral blood mononuclear cells obtained from healthy volunteers (target cells)

	RT-qPCR						ELISA		
Patient No.	FOXP3	FOXP3 PHA	GITR	GITR PHA	IFNγ	IFNγ PHA	IFNγ PHA	IL-5 PHA	IL-17 PHA
1	4.9	53.8	10.8	65.0	1.8	11.7	1580.0	8.4	0.0
2	8.2	65.4	18.7	175.9	3.8	30.5	37400.0	80.5	19.4
3	12.9	127.7	30.8	452.9	11.6	128.9	172000.0	187.0	239.0
4	9.2	153.7	32.5	399.2	5.0	52.0	97600.0	154.0	265.0
5	10.0	68.4	22.8	183.2	8.2	22.6	38100.0	81.5	118.0
6	4.0	4.6	8.9	55.2	1.3	10.4	0.0	0.5	42.7
7	11.5	36.7	22.4	107.0	6.1	19.3	1460.0	8.5	20.9
8	7.9	97.0	17.93	171.0	5.8	26.2	0.0	6.5	82.0
9	4.6	40.7	9.3	66.6	3.0	4.5	0.0	1.0	0.0
10	6.4	68.3	15.8	145.8	4.2	15.8	28.8	4.1	0.0
11	7.4	96.9	17.0	251.4	3.4	36.1	173.0	7.4	0.0
12	6.9	86.4	15.1	217.4	4.3	36.2	223000.0	144.0	638.0
13	5.2	32.7	14.1	83.0	3.8	5.3	11600.0	3.3	341.0
14	3.8	81.6	12.7	180.7	2.9	13.5	1660.0	0.5	181.0
15	5.4	31.2	9.9	54.4	3.6	3.3	16100.0	5.5	310.0

Secretion of interferon (IFN) γ, interleukin (IL)-5 and IL-17 from naive target cells was below detection limit. Phytohemagglutin (PHA) = Target cells activated with phytohemagglutin. RT-qPCR: Quantitative reverse transcriptase-polymerase chain reaction; GITR: Glucocorticoid-induced tumour necrosis factor receptor; FOXP3: Forkhead transcription factor 3.

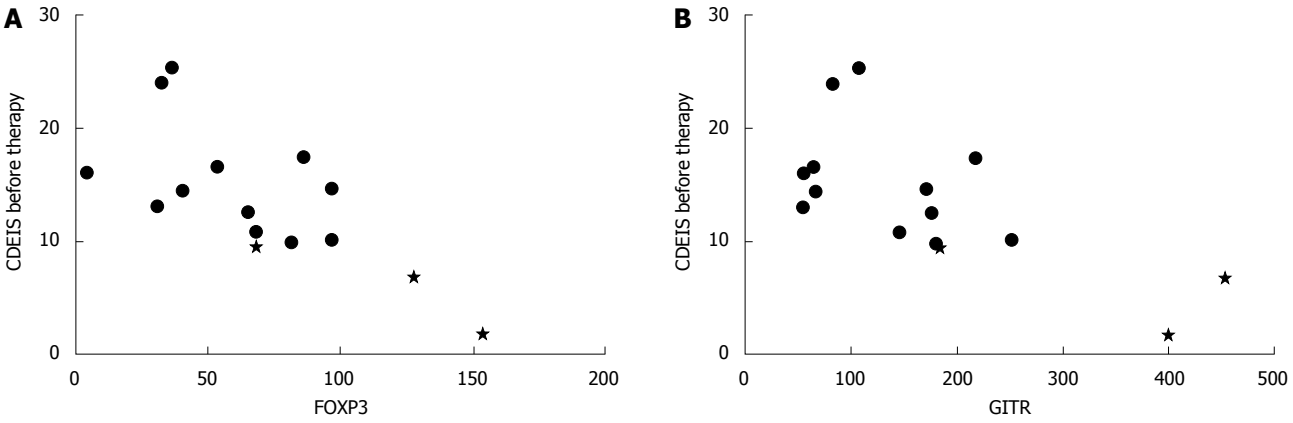

Figure 1 Patient serum withdrawn before anti-tumor necrosis factor-α therapy induced forkhead transcription factor 3 (A) and glucocorticoid-induced tumour necrosis factor receptor (B) specific mRNA expression (relative units) in activated target cells that correlated negatively with pre-treatment Crohn's disease endoscopic index of severity. [points; forkhead transcription factor 3 (FOXP3) r = -0.621, P = 0.013; glucocorticoid-induced tumour necrosis factor receptor (GITR) r = -0.625, P = 0.013]. Patients who had no decrease in Crohn's disease endoscopic index of severity (CDEIS) during therapy are marked with star.

= 0.241 for IFNγ, P = 0.286 for IL-5 and P = 0.980 for IL-17).

Correlations between target cell responses and the change of CDEIS during anti-TNF-α therapy

Low patient serum induced FOXP3, GITR and IFNγ specific mRNA expression in target cells was associated with a remarkable change of CDEIS observed during 3 mo therapy (FOXP3 r = -0.600, P = 0.018; GITR r = -0.589, P = 0.021; IFNγ r = -0.486, P = 0.066; Figure 2). Accordingly, in resting target cells GITR specific mRNA expression correlated with the change of CDEIS (r = -0.550, P = 0.034).

Also low serum induced IFNγ and IL-5 secretion from activated target cells was associated with a high change of CDEIS (r = -0.538, P = 0.039; r = -0.504, P = 0.055). IL-17 secretion from activated target cells did not correlate with the change of CDEIS (P = 0.467).

Findings related to fecal calprotectin

Fecal calprotectin decreased from a median of 1170 μg/g (range 88-15300) to a median of 130 μg/g (range 13-1400) within the 3 mo anti-TNF-α treatment (P = 0.001). No correlation was observed between target cell responses and calprotectin levels before or after treatment.

Findings related to ESR and CRP

ESR decreased from a median of 18 mm/h (range 6-58) to a median of 10.6 mm/h (range 1-40; P = 0.001) and CRP decreased from a median of 10 mg/L (range 0-54)

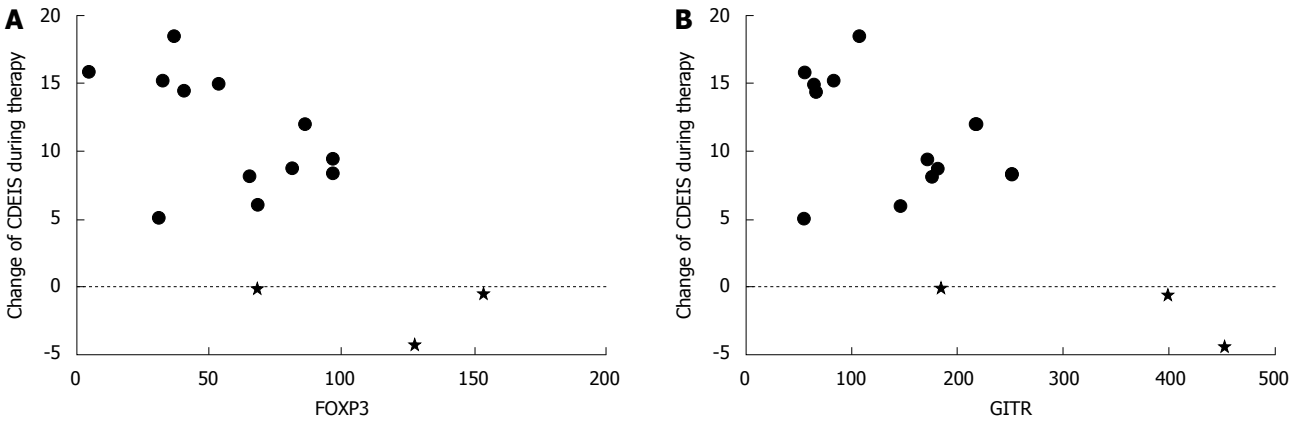

Figure 2 Patient serum withdrawn before anti-tumor necrosis factor-α therapy induced (A) forkhead transcription factor 3 (r = -0.600, P = 0.018) and (B) glucocorticoid-induced tumour necrosis factor receptor (r = -0.589, P = 0.021) specific mRNA expression (relative units) in activated target cells that had a negative correlation with the change of Crohn's disease endoscopic index of severity during three months therapy. The change of Crohn's disease endoscopic index of severity (CDEIS) corresponds with the decrease in points along improvement and is given as a positive value to illustrate the magnitude of therapeutic response. Patients who showed no decrease in the CDEIS during therapy are marked with star. GITR: Glucocorticoid-induced tumour necrosis factor receptor; FOXP3: Forkhead transcription factor 3.

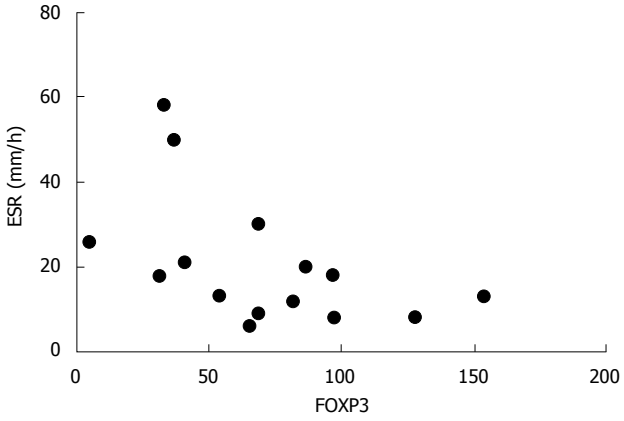

Figure 3 Patient serum withdrawn before anti-tumor necrosis factor-α therapy induced forkhead transcription factor 3 specific mRNA expression (relative units) in activated target cells that correlated negatively with erythrocyte sedimentation rate. ESR: Erythrocyte sedimentation rate. r = -0.548, P = 0.034.

to a median of < 5 mg/L (range < 5-11, P = 0.005) within 3 mo after introduction of anti-TNF-α therapy.

FOXP3 specific mRNA expression in activated target cells correlated negatively with pre-treatment ESR (r = -0.548, P = 0.034, Figure 3). There was no correlation between pre-treatment ESR and GITR ($P \geqslant 0.210$) or IFNγ ($P \geqslant 0.109$) specific mRNA expression or cytokine secretion from target cells (IFNγ P = 0.755, IL-5 P = 0.434, IL-17 P = 0.511).

FOXP3, GITR or IFNγ specific mRNA expression in target cells or IFNγ, IL-5 or IL-17 secretion from target cells did not correlate with the change of ESR or CRP during 3 mo anti-TNF-α therapy (all P = NS, data not shown).

DISCUSSION

We found that serum samples obtained from patients with CD before the introduction of anti-TNF-α therapy modulated the expression of regulatory T-cell (T-reg) markers FOXP3 and GITR and secretion of inflammatory cytokines IFNγ, IL-5 and IL-17 from target cells (peripheral blood mononuclear cells from healthy donors). The serum induced FOXP3, GITR and IFNγ responses of target cells correlated with the pre-treatment endoscopic status and also with therapeutic responses, i.e. mucosal improvement assessed with CDEIS within 3 mo.

In our previous study of children with IBD we found that the attenuation of systemic inflammation after the start of oral glucocorticoids was mirrored in the target cell responses induced by the patient serum[13]. We underline that we measured cytokine secretion and transcription markers of the target cells (donor derived PBMCs) modulated by the patient serum and not the activation stage of the PBMC from the patient. To our knowledge the ability of patient serum to activate signalling of normal T-cells has not previously been studied in adult CD patients at induction of anti-TNF-α therapy. It is remarked that post-treatment samples are not comparable here since the serum taken after the treatment contains an anti-TNF-α agent.

We found an inverse correlation between the expression of T-reg markers FOXP3 and GITR in target cells and the endoscopic disease activity before therapy. A similar inverse correlation existed also between FOXP3 and pre-treatment ESR. Since FOXP3 inhibits T-cell activation by its suppressive effect on transcription of cytokine genes[21] it is reasonable to assume that those patients whose serum environment seemed to mediate enhanced FOXP3 up-regulation as a response to T-cell stimulation had endoscopically milder disease. However, the serum induced expression of FOXP3 in target cells was not directly reflected in the cytokine activation of the target cells and cytokine response did not correlate with the pre-treatment CDEIS. Impaired up-regulation of GITR in the patient serum environment may be related to poor suppression of T-cell activation[22,23].

Interestingly, we also found that low expression of FOXP3 and GITR specific mRNA induced by patient serum obtained prior to therapy was associated with a good therapeutic response within 3 mo. These parameters were associated with high clinical activity and thus enhanced inflammation *in vivo*. High inflammatory activity at the early phase of anti-TNF-α therapy has been connected to the lack of therapeutic response in rheumatoid arthritis (RA). Previously non-responders to anti-TNF-α agents had a higher number of blood T-cells expressing chemokine receptors (CCR 3 and CCR 5) before the introduction of therapy[24]. In another study, high levels of serum IL-2 were associated with poor therapeutic response[25]. In our study, the group of patients that had high potency for FOXP3 induction in target cells showed poor clinical response to anti-TNF treatment. It was also evident that their disease activity before treatment was milder. TNF blocking has been shown to induce FOXP3 expression in patients with RA. Recovery of regulatory mechanisms has been proposed to be one of the mechanisms of action for TNF blocking[26]. It is possible that non-responders whose serum induced high FOXP3 up-regulation in target cells do not benefit from further activation of FOXP3 but their disease activity should be down-regulated by other mechanisms.

The majority of CD patients driven to anti-TNF-α therapy are on immunosuppressive medication such as azathioprine or methotrexate as here also. However, there was no correlation between patient serum induced expression or secretion of inflammatory cytokines from target cells and pre-treatment disease activity. We suggest that individual differences in target cell responses mediated by patient serum represents the net effect of maintenance medication, disease activity and patients immunological heterogeneity that together reflect the patients further capability to respond to biological therapy.

Fecal calprotectin correlates with the CDEIS and CRP[15,27]. In this study we failed to find statistically significant correlations between calprotectin or CRP and target cell responses. This finding was similar to the finding in our previous study of IBD children. Fecal calprotectin excretion reflects increased neutrophils and mononuclear cell migration into the gut lumen through the inflamed mucosa[28] and CRP is an acute phase protein produced predominantly in the liver in response to stimulation by IL-6, TNF-α and IL-1β[29]. It seems that the ability of patient serum to activate target cells reflects the immunological net effects in circulation in CD patients rather than inflammatory cell accumulation in the intestine reflected in fecal calprotectin.

To conclude, there are few studies of the mechanisms of treatment failure during anti-TNF-α therapy. An impaired response to anti-TNF-α therapy in CD has been suggested to be a result of early reactivation of the inflammatory cascade caused by individual intrinsic immunological mechanisms[10]. Also the inflammatory activity of the disease itself may play a role in the therapeutic response. We found that the immune activation potency of the patient serum that is monitored by gene expression profile of human PBMC is individual and correlates to later mucosal healing during anti-TNF-α therapy. Characterization of the key factors in serum that mediated the effects observed with this method, such as up-regulation of FOXP3 and GITR, could be one step toward better understanding of *in vivo* actions of anti-TNF-α therapies.

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COMMENTS

Background

Since the FDA approval of infliximab in 1999 for the treatment of severe Crohn's disease (CD), the use of tumor necrosis factor-α (TNF-α)-antagonist agents has emerged in treatment of severe inflammatory bowel disease. A significant proportion of patients, however, do not respond to the treatment. To date there are no means to foresee the therapeutic response or to monitor the response at an early phase of therapy with an TNF-α-antagonist agent.

Research frontiers

Recently, the authors applied a novel immunological assay for assessment of therapeutic response to glucocorticoids in pediatric patients with inflammatory bowel disease. In that study they showed that the therapeutic effect of corticosteroid therapy can be measured from patient serum at an early phase of the therapy. In the *in vitro* assay, a sample of patient serum is used to stimulate human white blood cells and the effect on specific white blood cell (T cell) markers is assessed. This kind of testing seems a promising means to predict individual responses to immunological therapies.

Innovations and breakthroughs

There are few studies exploring the immunological markers that reflect treatment response to anti-TNF-α therapy in CD. Here the authors used a recently described assay to measure therapeutic response to TNF-α-antagonist therapy from a patient serum sample. Disease activity and response to therapy is reflected in the patient serum and can be measured before the introduction of therapy. Serum induced changes in the specific white blood cell markers [forkhead transcription factor 3 (FOXP3), glucocorticoid-induced tumour necrosis factor receptor (GITR)] seemed to reflect individual response to anti-TNF-α therapy.

Applications

The results suggest that the effect of therapy with an anti-TNF-α agent can be measured from patient serum at an early phase. The study group was small and the results are preliminary, thus more studies are warranted to establish whether this kind of serum testing is suitable for predicting the individual response to anti-TNF-α therapy in clinical practice.

Terminology

Forkhead box P3, FOXP3, is a gene regulating the development and function of specific white cells, regulatory T cells. GITR is glucocorticoid-induced tumour necrosis factor receptor. FOXP3 and GITR are commonly used markers for regulatory T-cell activity.

Peer review

This study raises some interesting points never raised before about the T regulatory cell responses with biological agents.

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BRIEF ARTICLE

Nitric oxide activation of a potassium channel (BK_{Ca}) in feline lower esophageal sphincter

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Abstract

AIM: To assess the effect of nitric oxide (NO) on the large conductance potassium channel (BK_{Ca}) in isolated circular (CM) and sling (SM) muscle cells and muscle strips from the cat lower esophageal sphincter (LES) to determine its regulation of resting tone and relaxation.

METHODS: Freshly enzymatically-digested and isolated circular smooth muscle cells were prepared from each LES region. To study outward K^+ currents, the perforated patch clamp technique was employed. To assess LES resting tone and relaxation, muscle strips were mounted in perfused organ baths.

RESULTS: (1) Electrophysiological recordings from isolated cells: (a) CM was more depolarized than SM (-39.7 ± 0.8mV *vs* -48.1 ± 1.6 mV, $P < 0.001$), and maximal outward current was similar (27.1 ± 1.5 pA/pF *vs* 25.7 ± 2.0 pA/pF, $P > 0.05$); (b) The NO donor sodium nitroprusside (SNP) increased outward currents only in CM (25.9 ± 1.9 to 46.7 ± 4.2 pA/pF, $P < 0.001$) but not SM (23.2 ± 3.1 to 27.0 ± 3.4 pA/pF, $P > 0.05$); (c) SNP added in the presence of the BK_{Ca} antagonist iberiotoxin (IbTX) produced no increase in the outward current in CM (17.0 ± 2.8 *vs* 13.7 ± 2.2, $P > 0.05$); and (d) L-NNA caused a small insignificant inhibition of outward K^+ currents in both muscles; and (2) Muscle strip studies: (a) Blockade of the nerves with tetrodotoxin (TTX), or BK_{Ca} with IbTX had no significant effect on resting tone of either muscle; and (b) SNP reduced tone in both muscles, and was unaffected by the presence of TTX or IbTX.

CONCLUSION: Exogenous NO activates BK_{Ca} only in CM of the cat. However, as opposed to other species, exogenous NO-induced relaxation is predominantly by a non-BK_{Ca} mechanism, and endogenous NO has minimal effect on resting tone.

Key words: Circular smooth muscle; Feline; K^+ channel; Lower esophageal sphincter; Nitric oxide; Sling; Tone

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INTRODUCTION

Nitric oxide (NO) is the main neurotransmitter for active inhibition and relaxation of the lower esophageal sphincter (LES)[1-4]. In the esophageal body and in the LES circular muscle, NO activates the large conductance potassium channel (BK_{Ca}) and causes membrane hyperpolarization[5-9] as one potential mechanism leading to LES relaxation. NO has also been shown to be one putative neurotransmitter responsible for the membrane hyperpolarization of the inhibitory junction potential (IJP) in the LES circular muscle of the opossum, guinea pig, dog and mouse[6,10-15]. Activation of the BK_{Ca} by NO is a factor in production of the IJP membrane hyperpolarization[15].

In the cat, the BK_{Ca} channel plays a role in setting the resting membrane potential (RMP) in LES muscles[16]. This modulation of the RMP by the BK_{Ca} is also present in the opossum LES circular muscle and blockade of the BK_{Ca} results in an increase in tone attributed to a balance between the calcium-activated BK_{Ca} and chloride (Cl^-) channels that raise the RMP to the point where ongoing spiking activity is produced[17]. In the dog LES circular muscle, the activation of the BK_{Ca} in setting the RMP involves at least an endogenous source of NO[18,19]. It is not known to what extent endogenous NO is similarly involved in BK_{Ca} regulation of LES tone in other species.

The LES is composed of at least two separate muscle components, the circular and the oblique sling muscles[19]. In humans, the circular muscle forms only a partial ring (or semicircular clasp), whereas in other species including the cat, the circular muscle fully encircles the distal esophagus[19,20]. The circular and the sling muscles are functionally different with unique motor and electrophysiological properties. The circular muscle has significant spontaneous myogenic resting tone but is poorly responsive to cholinergic stimulation, whereas the sling muscle has little intrinsic myogenic tone but contracts vigorously to cholinergic stimulation, whereby it maintains its resting tone *in vivo*[21-26]. There are regional differences within the feline LES in terms of distribution, nature and function of ion channels[16,17,27-30]. In particular, the BK_{Ca} density is greater in circular muscle than in the sling muscle[16].

The objective of the present study was to determine in the cat, whether the BK_{Ca} in LES circular and sling muscles is influenced by exogenous and/or endogenous NO to regulate muscle resting tone and/or relaxation. These studies utilized isolated smooth muscle cells and strips taken from the feline LES circular and sling regions. The effect of endogenous *vs* exogenous sources of NO on the outward K^+ currents was also assessed and compared. A portion of this work has appeared in abstract form[31].

MATERIALS AND METHODS

Animal model

The cat was chosen as our animal model because of several important similarities between the cat and human esophagus. These similarities include (1) a significant portion of the distal esophageal body is composed of smooth muscle[20]; (2) the cholinergic sensitivity of the smooth muscle esophagus and LES are similar[22,23]; and (3) the placement of the gastroesophageal junction is similarly placed relative to the diaphragm[20].

Animal preparation

Experiments were approved by the University Health Network Animal Care Committee. Fasted, adult cats of either sex, weighing 2.5 to 5.0 kg were anesthetized with ketamine hydrochloride (0.15 mL/kg *im*; Bimeda-MTC, Cambridge, ON, Canada) and euthanized with pentobarbital sodium (0.5 mL/kg *iv*; Bimeda-MTC). At laparotomy, an esophago-gastric segment from 5 cm above the LES and including a 4 cm cuff of the stomach was carefully excised and placed into Krebs solution equilibrated with 95% O_2/5% CO_2 and maintained at pH 7.40 ± 0.05. The tissue was freed from surrounding fascia, stretched to its *in situ* length, and then cut along the greater curvature of the stomach. The mucosa was then gently removed to expose the LES circular and sling regions[22]. To consider regional differences within the LES circular *vs* sling muscles, muscle strips and isolated smooth muscle cells were prepared from each region.

Muscle strip studies

Muscle strips, 2 mm wide and 8 mm long, were obtained from the long axis of the circular and oblique sling muscles. Muscle strips were individually mounted in a 25 mL water-jacketed tissue bath. For isometric tension measurement (transducer model FT-03; Grass Instruments, Quincy, MA, USA) Force transducer data were acquired (Digi-Data 1200B analog-to-digital converter, Axon Instruments, Union City, CA, USA) and analyzed using pCLAMP software (version 8; Axon Instruments, Union City, CA, USA). Transmural electrical field stimulation (EFS) was delivered (Grass stimulator SP-9) through platinum wire electrodes with. 0.5 ms square-wave pulses in a 5 s train at a frequency of 10 Hz and a strength of 70 V.

Initially muscle strips were hung with 0.5 g tension for a 1 h equilibration period, and the length was then measured and defined as L_0 (initial length). Strips were then slowly stretched twice at increments of 25% of L_0 with 15 min between each stretch[24]. At study length of 150% L_0, EFS of the sling muscle resulted in an initial contraction in all strips and EFS of circular muscle resulted in relaxation in all strips studied[25].

The relative contribution of the BK_{Ca} channel to tension as affected by an exogenous or endogenous myogenic source of NO was assessed with the nerves intact or blocked with tetrodotoxin (TTX) and using the following protocols: (1) TTX alone (10^{-6} mol/L) or with blockade of the BK_{Ca} channel with iberiotoxin alone (IbTX, 10^{-7} mol/L), or the two in combination TTX (10^{-6} mol/L) + IbTX (10^{-7} mol/L); and (2) TTX (10^{-6} mol/L) + SNP [sodium nitroprusside (10^{-4} mol/L)] ± IbTX (10^{-7} mol/L) or TTX (10^{-6} mol/L) + IbTX (10^{-7} mol/L) ± SNP (10^{-4} mol/L). The chemicals were successively added and allowed 15-30 min to act, the strips were not washed in between experimental steps.

The data were normalized and expressed as tension: tension (mmol/L per mm^2) = [tone (g) × 9.81 m/s^2]/[cross-sectional area (mm^2)]; where the cross-sectional area (mm^2) = [tissue weight (mg)]/[1.05 mg/mm^3 × study length (mm)], and where 1.05 mg/mm^3 is the density of smooth muscle[32]. The unstimulated tension was referred to as baseline resting tension. After an experiment, each muscle strip was blotted onto a filter paper and weighed.

Isolated smooth muscle cell studies

Freshly enzymatically-digested and isolated circular smooth muscle cells were prepared from each LES region[30]. Two to three pieces (about 2 mm^2) of LES tissues were cut and placed into a test tube with 1 mL of dissociation solution and stored at 4℃ for up to 36 h. For cell dissociation of the cells, each of the following chemicals was added to the test tube: papain (2 mg/mL), collagenase Sigma blend type F (1.3 mg/mL), 1,4-dithio-L-threitol (154 μg/mL) and bovine serum albumin (1 mg/mL) were added and the tube was incubated at 35℃ for 30-60 min. Following rinsing in enzyme-free dissociation solution and gentle mechanical agitation, isolated spindle-shaped single smooth muscle cells were allowed to settle and adhere to a 1 mL glass bottom recording chamber, mounted on the stage of an inverted microscope. After 30 min, cells were washed with external solution and used within 4 h of isolation. Recordings were performed at room temperature (22-25℃).

To study outward K^+ currents, the perforated patch clamp technique was employed, with the pipette tip resistance between 2-4 MΩ. The pipettes were front-filled with the pipette solution and back-filled with the nystatin solution. With a giga ohm seal the cell was held at -50 mV and a whole-cell configuration was achieved within 5 min. Once a stable access resistance (i.e. below 20 MΩ) had been achieved and maintained, the RMP was measured. To study the effect of NO, two approaches were employed: (1) puffing sodium nitroprusside (SNP, NO donor, 10^{-4} mol/L) onto the muscle cell with a picospritzer[29] positioned 100 μm from the cell (to mimic NO release from nerves); and (2) adding N^G-nitro-L-arginine (L-NNA; NO synthase inhibitor; 10^{-4} mol/L) to the recording dish (to assess the potential contribution of endogenous NO). BK_{Ca} channel currents were blocked by the addition of iberiotoxin (IbTX, 200 nmol/L).

To record outward K^+ currents, voltage-ramp protocols (starting and ending with the holding potential of -50 mV), from -140 to +60 mV or from -70 to +70 mV over 500 ms were generated by a pClamp 9.0 software (Axon Instruments, Union City, CA,USA) and recorded by an Axopatch 200B amplifier (Axon Instruments). Before digitization, all signals were sampled at 10 kHz, filtered at 1 kHz by an on board eight-pole Bessel filter, and digitized (Digidata 1320 converter, Axon Instruments). Cell capacitance was determined by integration of the capacitance transient, and the maximal current density was normalized for cell size. Peak outward currents were compared after each pharmacological manipulation. Recordings were analyzed using Clampfit 9.0 software (Axon Instruments).

Chemicals and solutions

Atropine (atropine sulfate), TTX (Alomone Labs, Jerusalem, Israel), IbTX (Alomone Labs) and the dissociation enzymes were reconstituted into deionized distilled water. SNP was diluted into the external solution. Seventy-five mg of L-NNA was added into 5 mL of 0.1 mol/L HCl, heated gently until dissolved, and then brought up to 10 mL with water. For nystatin, 0.006 g of the powder was dissolved into 200 μL DMSO and 13 μL of that stock was further diluted into 2 mL of pipette solution. If not otherwise stated, all chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

For the muscle strip studies, the Krebs solution (115 mmol/L NaCl, 4.6 mmol/L KCl, 1.2 mmol/L $MgSO_4 \cdot 7H_2O$, 1.2 mmol/L $NaH_2PO_4 \cdot H_2O$, 22 mmol/L $NaHCO_3$, 2.0 mmol/L $CaCl_2 \cdot 2H_2O$ and 11 mmol/L dextrose) was maintained at 37℃ with 95% O_2/5% CO_2 in the organ bath. For the isolated smooth muscle studies, the dissociation solution (135 mmol/L KCl, 10 mmol/L HEPES, 10 mmol/L glucose, 10 mmol/L taurine, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 0.25 mmol/L EDTA; pH 7.0 adjusted with 1 mol/L KOH), the external solution (130 mmol/L NaCl, 5 mmol/L KCl, 20 mmol/L HEPES, 10 mmol/L glucose, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$; pH 7.4 adjusted with 1 mol/L NaOH) and the pipette solution (140 mmol/L KCl, 20 mmol/L HEPES, 10 mmol/L glucose, 0.1 mmol/L EGTA, 1 mmol/L $MgCl_2$; pH 7.2 adjusted with 1 N KOH) all had an osmolarity between 285 and 310 mOsm.

Statistical analysis

All data are expressed as mean ± SE, where "*n*" represents the number of muscle strips (*n* = 6-11) or cells (*n* = 6-13) studied per group of 2-5 cats per experiment. Each muscle strip or cell served as its own control. SAS software (SAS Institute, Cary, NC, USA) was used to determine statistical differences between groups by a repeated measure ANOVA, followed by a *post hoc* Bonferroni adjusted paired-wise comparison test. A Student's *t*-test was also used to determine statistical differences between means. An alpha value of 0.05 was considered significant.

RESULTS

Effects of NO on the BK_{Ca} channel in LES isolated smooth muscle cells

The RMP of the LES circular smooth muscle cells (no added drugs) was significantly more depolarized than the sling smooth muscle cells (-39.7 ± 0.8 mV *vs* -48.1 ± 1.6 mV, $P < 0.001$, data not shown). Ramp protocols elicited outward potassium currents at potentials greater than -35 mV. The maximal total outward current was similar in isolated smooth muscle cells from the LES circular and sling regions (27.1 ± 1.5 pA/pF *vs* 25.7 ± 2.0 pA/pF, $P > 0.05$).

To mimic the effect of NO release from nerves, the NO donor, SNP, was puffed onto the smooth muscle cells, and a ramp protocol from -140 to +60 mV was used. SNP increased outward potassium currents of LES circular

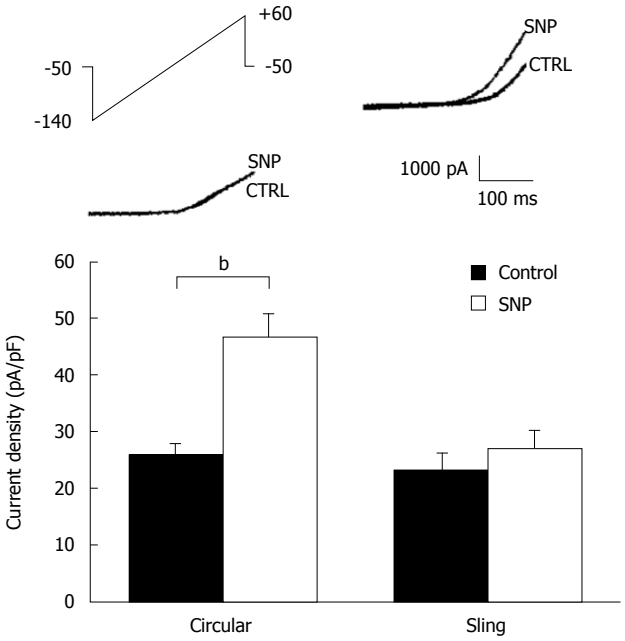

Figure 1 Representative traces (last 300 ms of trace shown) and quantification of the effect of sodium nitroprusside (10^{-4} mol/L) on elicited outward K^+ currents using a ramp protocol from -140 to +60 mV of lower esophageal sphincter circular (n = 13) and sling (n = 8) smooth muscle cells. The maximal total outward current was similar in isolated smooth muscle cells from the lower esophageal sphincter (LES) circular and sling regions. The nitric oxide donor sodium nitroprusside (SNP) increased outward potassium currents of LES circular smooth muscle cells. In contrast, SNP did not significantly cause an increase in the outward K^+ currents in LES sling muscle. [b]$P < 0.001$.

smooth muscle cells from 25.9 ± 1.9 to 46.7 ± 4.2 pA/pF ($P < 0.001$, Figure 1); an 80% increase, and hyperpolarized the cells (from -42.2 ± 1.3 to -63.4 ± 5.0 mV, $P < 0.05$, Table 1). In contrast, SNP did not cause a significant increase in the outward K^+ currents (from 23.2 ± 3.1 to 27.0 ± 3.4 pA/pF, $P > 0.05$, Figure 1) of sling cells, and no significant change in the RMP (Table 1) was observed. SNP also activated outward potassium currents in circular smooth muscle from both proximal (21.9 ± 3.0 to 38.5 ± 9.6 pA/pF, n = 5) and distal (23.5 ± 2.3 to 39.1 ± 6.0 pA/pF, n = 8) esophageal body sites.

In the LES, since the effect of the exogenous NO donor (SNP) was seen only in the circular muscle cells, we then pharmacologically isolated the portion of the recorded outward potassium currents attributed to the BK_{Ca} channel currents only in the circular muscle cells[16]. The BK_{Ca} channel was blocked with 200 nmol/L IbTX, and a voltage-ramp protocol, from -140 to +60 mV, was employed where the different steps of the protocol were performed on the same LES circular smooth muscle cells. SNP induced a significant increase in elicited outward potassium currents *vs* control current density (37.9 ± 4.9 pA/pF *vs* 26.7 ± 3.5 pA/pF, $P < 0.05$, n = 8, Figure 2). IbTX alone blocked a significant portion of the control outward potassium currents (13.7 ± 2.2 pA/pF *vs* 26.7 ± 3.5 pA/pF, $P < 0.05$, n = 8, Figure 2). SNP added in the presence of IbTX, produced no significant increase in the outward currents (17.0 ± 2.8 *vs* 13.7 ± 2.2, $P > 0.05$ for IbTX+SNP *vs* IbTX alone, n = 8, Figure 2). In separate experiments, blockade of K_{DR} with

Table 1 Resting membrane potential (mV) of isolated cells from the lower esophageal sphincter circular and sling regions in the absence or presence of sodium nitroprusside or L-NNA

	Circular	Sling
Control	-42.2 ± 1.3	-53.0 ± 1.2
Sodium nitroprusside	-63.4 ± 5.0[a]	-50.3 ± 3.1
Control	-40.5 ± 1.4	-44.0 ± 1.1
L-NNA	-33.8 ± 1.5[a]	-38.8 ± 2.3

[a]$P < 0.05$ *vs* control.

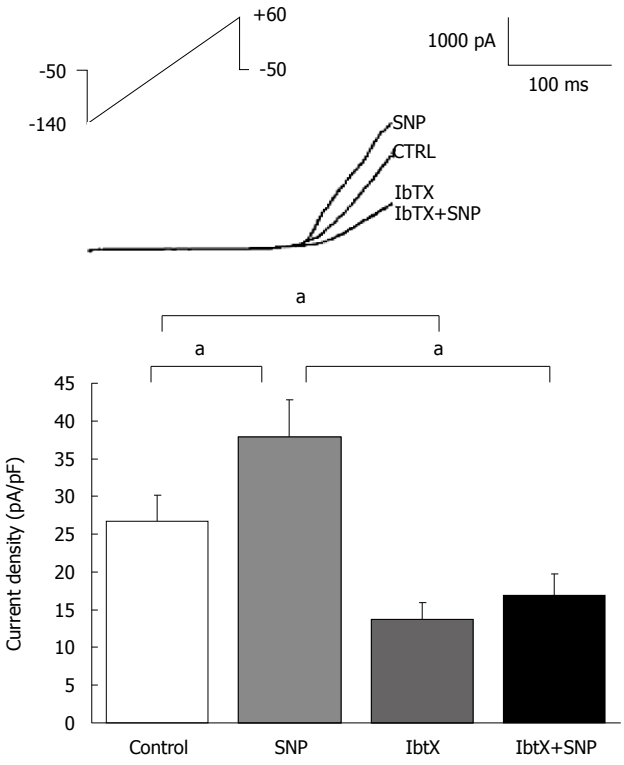

Figure 2 Pharmacological identification, with iberiotoxin (200 nmol/L), of the portion of the elicited outward K^+ currents, using a ramp protocol from -140 to +60 mV, corresponding to BK_{Ca} channel currents in lower esophageal sphincter circular (n = 8) smooth muscle cells. Last 300 ms of representative traces shown. Sodium nitroprusside (SNP) induced a significant increase in elicited outward potassium currents *vs* control current density. Iberiotoxin (IbTX) alone blocked a significant portion of the control outward potassium currents. SNP added in the presence of IbTX, produced no significant increase in the outward currents. [a]$P < 0.05$.

DTX (200 nmol/L) did not inhibit SNP induced outward potassium currents. Thus, for the circular smooth muscle cells, SNP, an exogenous source of NO, produced a significant activation of the BK_{Ca} channel.

To assess the potential contribution of an endogenous source of NO in the two LES regions, the NO synthase inhibitor, L-NNA, was added and a voltage-ramp protocol from -70 to +70 mV was used. Addition of L-NNA led to an insignificant 14% decrease in outward potassium currents in the circular smooth muscle cells (from 29.1 ± 2.6 to 25.0 ± 2.4 pA/pF, $P > 0.05$, Figure 3), however, L-NNA significantly depolarized the cell (from -40.5 ± 1.4 to -33.8 ± 1.5 mV, $P < 0.05$, Table 1), a 17% change in the

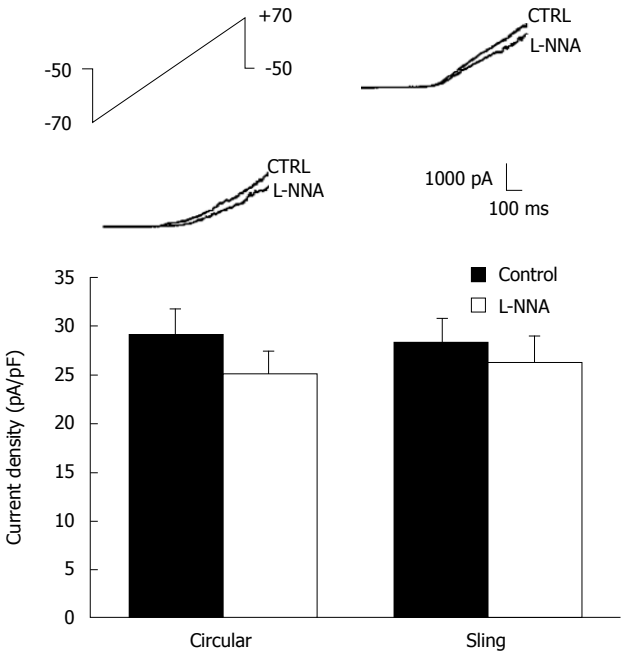

Figure 3 Representative traces and quantification of the effect of L-NNA (10^{-4} mol/L) on elicited outward K^+ currents using a ramp protocol from -70 to +70 mV of lower esophageal sphincter circular (*n* = 8) and sling (*n* = 8) smooth muscle cells. Addition of L-NNA led to an insignificant 14% decrease in outward potassium currents in the circular smooth muscle cells. In the sling smooth muscle cells, the addition of L-NNA led to an insignificant 7% reduction in current density.

RMP. In the sling smooth muscle cells, there was a smaller reduction in current density (from 28.2 ± 2.5 to 26.3 ± 2.7 pA/pF, $P > 0.05$, Figure 3), a 7% decrease, and only a 3% increase in the RMP (from -44.0 ± 1.1 to -38.8 ± 2.3 mV, $P > 0.05$, Table 1). Thus, an endogenous source of NO may modulate outward potassium currents and RMP, but to a small extent, and the effect is more pronounced in the circular muscle.

Effects of NO on the BK_{Ca} channel in LES smooth muscle strips

In view of the small effect of endogenous NO on the RMP of both circular and sling muscle cells, we investigated the myogenic contribution of the BK_{Ca} channel to basal resting smooth muscle tone. Nerves were blocked with TTX and the BK_{Ca} channel was inhibited with IbTX. For the circular muscle, TTX (tension 14.5 ± 3.4 mmol/L per mm^2 *vs* 18.2 ± 4.7 mmol/L per mm^2) or IbTX (tension 14.5 ± 2.3 mmol/L per mm^2 *vs* 15.0 ± 2.3 mmol/L per mm^2) did not significantly change the resting tension ($P > 0.05$, Figure 4A). The combined blockade of the nerves and the BK_{Ca} channel also resulted in no significant change in the overall muscle tone (7.3 ± 1.3 mmol/L per mm^2 *vs* 8.3 ± 1.1 mmol/L per mm^2, $P > 0.05$, Figure 4A). Similarly, for the sling muscle, TTX (tension 10.3 ± 2.0 mmol/L per mm^2 *vs* 10.5 ± 2.4 mmol/L per mm^2) or IbTX (tension 7.3 ± 1.2 mmol/L per mm^2 *vs* 7.3 ± 1.3 mmol/L per mm^2) or TTX and IbTX together (tension 6.3 ± 1.5 mmol/L per mm^2 *vs* 8.4 ± 2.4 mmol/L per mm^2) did not significantly modulate the basal resting tone ($P > 0.05$, Figure 4B).

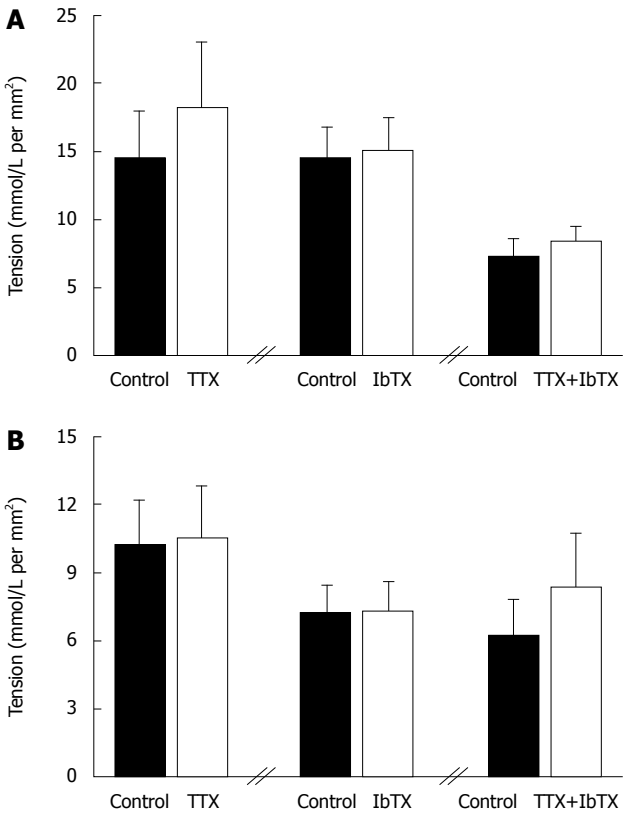

Figure 4 Effect of the blockade of the BK_{Ca} channel with iberiotoxin (10^{-7} mol/L) of lower esophageal sphincter (A) circular (*n* = 6-8) and (B) sling (*n* = 7-11) smooth muscle tone with intact nerves or blocked with tetrodotoxin (10^{-6} mol/L). A: For the circular muscle, tetrodotoxin (TTX) or iberiotoxin (IbTX) did not significantly change the resting tension ($P > 0.05$). The combined blockade of the nerves and the BK_{Ca} channel also resulted in no significant change of the overall muscle tone ($P > 0.05$); B: Similarly, for the sling muscle, TTX, IbTX or TTX and IbTX together did not significantly modulate the basal resting tone ($P > 0.05$). //: Separates different experiments.

To study the effect of exogenous NO on the BK_{Ca} channel in muscle strips, SNP was added in the presence or absence of IbTX, and *vice versa*, with the nerves blocked with TTX. In LES circular muscle, SNP decreased the resting tension by 17.4 ± 5.7 mmol/L per mm^2, the tension was unchanged with further addition of IbTX ($P > 0.05$, Figure 5). When IbTX was added first, there was no significant change in tension, while the subsequent addition of SNP (IbTX+SNP in the presence of TTX) significantly decreased the change in tension (1.1 ± 0.5 mmol/L per mm^2 *vs* -6.3 ± 1.2 mmol/L per mm^2, $P < 0.001$, Figure 5). For the sling muscle, similar changes were observed. SNP or SNP+IbTX decreased the tension to the same extent, IbTX alone produced no significant change in resting tension, and IbTX+SNP decreased the tension by 4.1 ± 1.0 mmol/L per mm^2 ($P < 0.001$, Figure 5). Therefore, in the cat, whether the BK_{Ca} channel is blocked or not, exogenous NO causes relaxation in both sling and circular smooth muscles.

The effects of IbTX on neural responses induced by Electrical Field Stimulation (EFS) were assessed. The addition of TTX was omitted in these studies. In circular muscle, EFS caused transient relaxation of resting tone

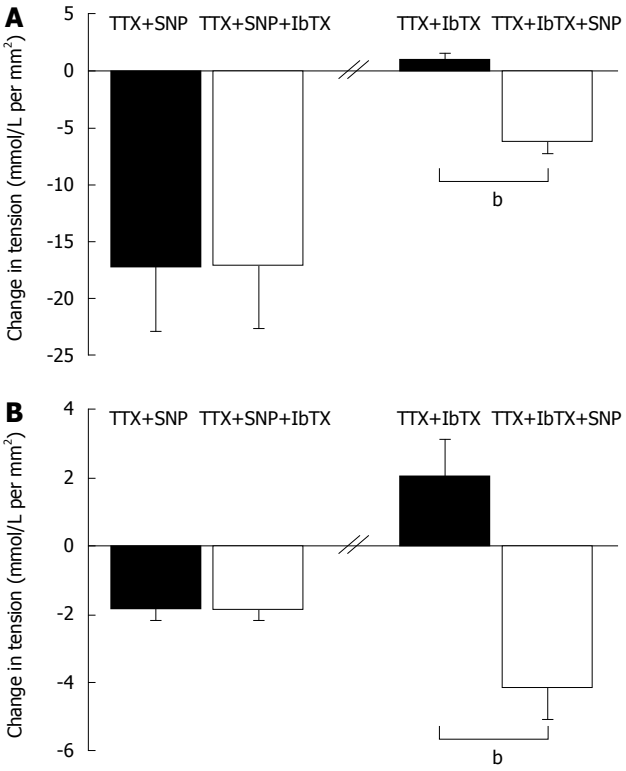

Figure 5 Effect of sodium nitroprusside (10^{-4} mol/L) ± iberiotoxin (10^{-7} mol/L), and iberiotoxin (10^{-7} mol/L) ± sodium nitroprusside (10^{-4} mol/L) on smooth muscle tone relative to basal tone in the presence of tetrodotoxin (10^{-6} mol/L) in muscle strips of lower esophageal sphincter (A) circular (*n* = 6) and (B) sling (*n* = 6-9) muscles. A: In lower esophageal sphincter (LES) circular muscle, sodium nitroprusside (SNP) decreased the resting tension and this tension was then unchanged with further addition of iberiotoxin (IbTX) ($P > 0.05$). When IbTX was added first, there was no significant change in tension, while the subsequent addition of SNP [IbTX+SNP in the presence of tetrodotoxin (TTX)] significantly decreased the tension ($P < 0.001$); B: In LES sling muscle, SNP or SNP+IbTX decreased the tension to the same extent, and IbTX alone produced no significant change in resting tension. IbTX+SNP decreased the tension ($P < 0.001$). //: Separates different experiments. $^bP < 0.001$.

from 14.5 ± 2.3 to 3.8 ± 1.9 mmol/L per mm^2, and IbTX had no effect on LES tone (15.0 ± 2.3 *vs* 14.5 ± 2.3, $P > 0.05$) or EFS induced LES relaxation (3.9 ± 1.9 mmol/L per mm^2 *vs* 3.8 ± 1.9 mmol/L per mm^2, $P > 0.05$). In the LES sling muscle, EFS caused a transient contractile response from resting tone of 7.3 ± 1.2 to 19.3 ± 4.1 mmol/L per mm^2. IbTX had no effect on resting tone (7.3 ± 1.3 mmol/L per mm^2 *vs* 7.3 ± 1.2 mmol/L per mm^2, $P > 0.05$) or EFS induced responses (19.3 ± 4.1 mmol/L per mm^2 *vs* 23.4 ± 5.8 mmol/L per mm^2, $P > 0.05$).

DISCUSSION

In view of the part played by the activation of the BK_{Ca} by NO in the modulation of resting tone and relaxation of the LES in some species, the present study explored this role in the sling and circular muscle components of the cat LES. The BK_{Ca} responsiveness to the inhibitory neurotransmitter NO was significant only in the LES circular muscle. Furthermore, exogenous NO, whether provided by SNP or released by EFS, produced its inhibitory effect to relax LES smooth muscle predominantly through other mechanisms than the activation of the BK_{Ca} channel. There may be a small endogenous source of NO that has a small inhibitory effect to slightly reduce resting tone in both muscles. These findings add to the other known physiological properties of the two muscles and their regional differences. Any NO-BK_{Ca} relaxant effect, if needed, would be directed primarily to facilitate relaxation of the high intrinsic myogenic tone of the LES circular muscle.

Since SNP produced a large and significant activation of the BK_{Ca} channel only in the circular smooth muscle cells, a difference in sensitivity of the BK_{Ca} to NO is one possible explanation for this disparity, assuming that the two muscles express a similar number of channels[33]. This disparity could also reflect regional differences in K^+ channel molecular constitution through expression of different regulatory subunits leading to channel isoforms with different channel activity and sensitivity to voltage and intracellular calcium[15,34]. In the human esophagus there are at least four different splice variants of BK_{Ca} with differences in expression at various sites of the esophageal body and LES[33].

It is generally accepted that *in vivo*, NO is the main neurotransmitter for active inhibition of the LES[1-4]. The circular muscle with its high intrinsic tone relaxes predominantly due to the neural release of the inhibitory neurotransmitter NO as well as some non-NO inhibitory influence[2,3,9,13,25,35-39]. The sling muscle with its tone maintained by cholinergic excitation[21-24,26,40], presumably relaxes predominantly by switching off this excitatory influence, although NO can also relax the sling when contracted by cholinergic stimulation[24,30].

The primary mechanism for NO-mediated relaxation is considered to be *via* a cyclic guanosine monophosphate (cGMP) pathway[41-43]. There are several potential intracellular pathways whereby NO can lead to smooth muscle relaxation. Activation of the BK_{Ca} by NO could potentially occur directly or indirectly through interaction with a number of these mechanisms. For example, the PKG-pathways are accepted intracellular messengers that can lead ultimately to LES circular muscle relaxation in dog, opossum and cat[44-51]. In addition, an involvement of the contractile proteins has been proposed in the sequence of events targeted by NO for LES relaxation in opossum[14]. The BK_{Ca} channel integrates changes in both intracellular Ca^{2+} and membrane potential, and the cGMP pathway can in counter-part influence these latter parameters[15]. Moreover, cross-talk between NO and the ion channel may be facilitated by a close spatial relationship between ion channels and NOS sequestered in or in the vicinity of caveolae, seen in dog LES circular muscle[52-54]. Although it is unclear why endogenous NO had minimal effect on resting LES tone in this study of the cat, variability in the effect of different NO donors and NO states on gut smooth muscle physiology as well as species differences have also been reported and are likely contributing factors[55]. The apparent species and smooth muscle differences open the door to further experimentation. Our aim in this paper was not

directed to a study of the potential intracellular pathways and mechanisms underlying these differences.

Although the BK_{Ca} current density is greater in the circular muscle, the channel plays a role in setting the RMP in both LES muscles[16]. IbTX causes significant membrane depolarization in both LES muscles, as confirmed in the present study. This modulation of the RMP by the BK_{Ca} is also present in the opossum LES circular muscle[17]. Our present experiments in isolated smooth muscle cells of the cat LES, show that this activation of the BK_{Ca} in setting the RMP involves at least an endogenous source of NO in both LES regions, consistent with findings in dog LES circular muscle[9,18].

Although the BK_{Ca} contributes to setting the RMP, and[16,17], in the current muscle strip studies, blockade of the BK_{Ca} with IbTX did not result in a significant increase in muscle tension in either muscle. This is unlike the esophageal body where both IbTX and tetra ethyl ammonium increase tonic and phasic contractility[8]. In the opossum LES circular muscle, blockade of the BK_{Ca} results in an increase in tone when the RMP is raised to the point where ongoing spiking activity is produced. In those experiments, BK_{Ca} blockade changed the RMP only slightly (from -43.4 to -37.8 mV). The further transient depolarizations associated with the spike-like action potentials then are associated with calcium (Ca^+) entry through the L-type channel[17]. The findings fit with the knowledge that Ca^{2+} is required for, and Ca^{2+} availability can alter LES circular smooth muscle motor response in dog and opossum[7,14,18,56].

The lack of major effect of IbTX on resting tone in both cat LES muscles in the present experiments would occur if membrane depolarization is unable to sufficiently activate channels for entry of extracellular calcium for tension development, either directly or through the stimulation of spiking activity. In the LES muscles, L-type calcium channel activation is only seen at membrane potentials more positive than about -20 mV[27,57], levels not reached in the sling (-42 mV) or in the circular muscle (-31 mV) with IbTX or other blockade of the BK_{Ca}[16,17]. Furthermore, we have shown that resting tone in both muscles utilizes both intracellular and extracellular calcium sources, the circular muscle more dependent on an extracellular, and the sling on an intracellular source[28]. In addition, the two muscles utilize different calcium entry portals, the circular muscle using the L-type and the sling a non L-type channel. Therefore, our findings in the cat indicate that if endogenous NO is acting on the BK_{Ca} to modulate resting tone, this modulation is mainly through an indirect action, and augmented by its other mechanisms of action[7,14,15,18,49]. Furthermore, there appears to be a more complex relationship in the LES between active changes in membrane potential such as spiking activity or slow wave activity and levels of LES tone[58-63]. Our present findings, taken together with findings in other species, indicate that changes in resting tone are likely dependent on Ca^{2+} entry associated with the active changes in membrane potential rather than entry with any small changes in the RMP[17]. Further studies are required to assess which mechanisms and pathways are involved.

In terms of resting tone in the LES, our findings have potential functional significance. In the present *in vitro* study in the cat, TTX blockade of excitatory as well as inhibitory nerve activity failed to significantly affect basal tension in circular smooth muscle strips. This finding supports the concept that *in vivo*, intrinsic ongoing myogenic contraction underlies resting tone of the circular muscle. The intrinsic tone in the circular muscle is not significantly modulated by cholinergic excitation and/or nitrergic inhibition[3,4]. On the other hand, cholinergic neural influence, normally vagally-driven, acts to augment and maintain the low intrinsic basal resting tone in the sling.

A better understanding of the asymmetries of both circular and sling LES muscles at the cellular level could give insights into the pathogenesis of and potential therapeutic usefulness for patients with motor disorders such as achalasia and gastroesophageal reflux disease. For instance, in human and cat, addition of atropine decreases the leftward resting LES pressure where the sling is located, with little effect in the other radial orientations[22,40]. Although in the present cat experiments, exogenous NO causes smooth muscle relaxation of both the LES circular and sling smooth muscles, whether the BK_{Ca} channel is blocked or not, activation of the BK_{Ca} channel by NO provides another potential mechanism to augment the muscle relaxing effect of the cGMP pathway. Whether such a mechanism may become more important, for example in circumstances where NO release to produce relaxation of the circular muscle is decreased, as in patients with achalasia, requires further study[64-68]. Hence, regional differences in muscle cell BK_{Ca} responsiveness to NO hold the potential for selective modulation of LES function in health and disease.

COMMENTS

Background

The lower esophageal sphincter (LES) is normally closed to prevent reflux of gastric acid, but opens with a swallow to allow passage of the bolus into the stomach. The LES has two main components, the circular and sling muscles. To maintain closure, the circular muscle has significant resting tone but is poorly responsive to neural cholinergic excitation. On the other hand, the sling has little resting tone, and its closing contraction is maintained by vagal release of acetylcholine. Opening of the LES with a swallow requires relaxation of the two muscles. Relaxation of the circular muscle is therefore dependent on active relaxation induced by an inhibitory neurotransmitter such as nitric oxide (NO), while relaxation of the sling can readily occur with vagal excitation turned off.

Research frontiers

Although NO can relax smooth muscle by a number of cellular mechanisms, in some species activation of the BK_{Ca} by NO is important for the modulation of resting tone and relaxation of the LES. The importance of this mechanism was assessed in isolated cells and in strips of muscle from the two muscle components of the cat LES. The cat esophagus is very similar to the human esophagus with a significant portion of the distal esophagus composed of smooth muscle.

Innovations and breakthroughs

The BK_{Ca} responded to the inhibitory neurotransmitter NO, administered as sodium nitroprusside (SNP), only in the LES circular muscle, the muscle with high resting tone. Furthermore and as opposed to other species, exogenous NO, whether provided by SNP or released by electrical field stimulation, provided its inhibitory effect to relax LES smooth muscle predominantly through other mechanisms than the activation of the BK_{Ca} channel.

Applications

Any NO-BK_{Ca} relaxant effect, if needed, would be directed primarily to facilitate

relaxation of the high intrinsic myogenic tone of the LES circular muscle. Such a mechanism may become more important, for example in circumstances where NO release to produce relaxation of the circular muscle is decreased, as in patients with achalasia. Since NO provided its inhibitory effect to relax LES smooth muscle predominantly through other mechanisms than the activation of the BK_{Ca} channel, further research should be directed to these other mechanisms. Such studies hold the potential for new therapeutic approaches applicable to the human esophagus.

Terminology

Achalasia: a condition where swallowing difficulty is due to obstruction of the esophagus at the LES resulting from failure of LES relaxation due to absence of the inhibitory neurotransmitter NO.

Peer review

This is a well-designed and interesting study that provides convincing evidence regarding the activation of large conductance potassium channels within the circular smooth muscle during administration of exogenous nitric oxide. The role of endogenous nitric oxide appears to be relatively less significant.

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BRIEF ARTICLE

Effects of meal size and proximal-distal segmentation on gastric activity

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Abstract

AIM: To evaluate the effects of meal size and three segmentations on intragastric distribution of the meal and gastric motility, by scintigraphy.

METHODS: Twelve healthy volunteers were randomly assessed, twice, by scintigraphy. The test meal consisted of 60 or 180 mL of yogurt labeled with 64 MBq ^{99m}Tc-tin colloid. Anterior and posterior dynamic frames were simultaneously acquired for 18 min and all data were analyzed in MatLab. Three proximal-distal segmentations using regions of interest were adopted for both meals.

RESULTS: Intragastric distribution of the meal between the proximal and distal compartments was strongly influenced by the way in which the stomach was divided, showing greater proximal retention after the 180 mL. An important finding was that both dominant frequencies (1 and 3 cpm) were simultaneously recorded in the proximal and distal stomach; however, the power ratio of those dominant frequencies varied in agreement with the segmentation adopted and was independent of the meal size.

CONCLUSION: It was possible to simultaneously evaluate the static intragastric distribution and phasic contractility from the same recording using our scintigraphic approach.

Key words: Gastric motility; Phasic contractions; Proximal stomach; Scintigraphy; Meal size

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INTRODUCTION

Scintigraphy is the gold standard for measuring gastric emptying and offers the advantage of completely characterizing the complex physiology of intragastric distribution of the meal (IDM) between the proximal and the distal regions[1-3]. Quantification of intragastric distribution could help to define abnormal physiology and explain certain functional dyspeptic symptoms[4], especially when global gastric emptying values are normal[3]. Proximal-distal segmentation approaches employed to divide the stomach in proximal and distal regions remain a challenge for studying IDM; therefore, a validation is necessary before incorporating it into clinical practice[3].

At least three segmentation approaches were adopted, based on the proximal stomach defined immediately after a meal, to divide the stomach into two equal areas and using the incisura[1,2,4-7]. No previous studies have compared the results obtained by those three approaches, and several studies have perceived that there may be problems regarding the division of gastric segments into proximal and distal regions[6,8]. In particular, the two gastric compartments might not be easily identifiable, the incisura may not be pronounced, and somewhat arbitrary definitions cannot be always applied[8].

Apart from the well-known phasic motor activity of 3 cpm (cycle per minute) in the distal stomach, a phasic activity of 1 cpm was observed in dogs and humans using different techniques and appears to be concentrated in the proximal region[9-12]. Thus, considering that each frequency of contraction can be associated with one gastric region, the dominant frequencies could facilitate the characterization of the proximal and distal regions of stomach. The functions of the stomach regions can vary according to the nutrient content[7], and there is a clinical recommendation for consumption of smaller and more frequent meals to avoid postprandial symptoms in patients with common gastrointestinal disorders[13,14]. However, there is little information about the effects of meal size on intragastric distribution, especially for semisolid small meals. The aim of this study was to evaluate the effects of meal size and three proximal-distal segmentations on intragastric distribution and gastric motor activity by scintigraphy.

MATERIALS AND METHODS

Subjects

Twelve healthy volunteers (three female and nine male) with a range of body mass indices of 18.5-24.9 kg/m^2 and an age of 25-45 years participated in the studies. None had a history of digestive disease or abdominal surgery. Informed written consent was obtained from each participant. The studies were performed in agreement with Declaration of Helsinki and the local Ethics Committee approved the protocol.

Study protocol

Each volunteer was evaluated twice on separate occasions for ingestion of 60 and 180 mL of a semisolid test meal (yogurt containing 1 kcal/mL). The yogurt was chosen to simulate a small meal that is commonly ingested between large meals. *In vitro* tests were achieved to assure that the Tc-99m tin colloid and the yogurt were adequately blended[12]. The studies were performed in the morning, after an overnight fast, in a randomized order, and were separated by an interval of one wk. Both test meal were labeled with 64 MBq ^{99m}Tc-tin colloid as a nonabsorbable carrier and consumed with the volunteers standing upright in front of the gamma camera.

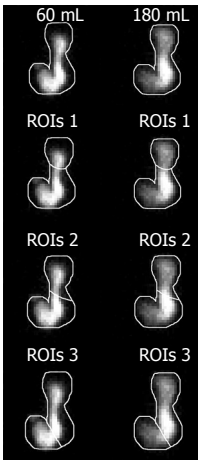

Figure 1 Scintigraphic images showing the composite images after meals with 60 mL and 180 mL, and the three proximal-distal image segmentations (ROIs) adopted. ROIs: Regions of interest.

A dual-head gamma camera (Sopha Vision, Model DST, Sophycammera; Medical Sopha Vision America, Twinsburg, OH, USA) equipped with a parallel-hole low-energy and high-resolution collimator was used. The gamma camera was set up to record activity around the 140-keV photopeak of ^{99m}Tc. A dynamic set of 1080 frames (1 frame/s) was acquired for 18 min and images were stored in a 64 × 64 matrix for further analysis. A geometric mean of the anterior and posterior gastric counts was determined for each time point and corrected for radionuclide decay[3].

Data analysis

All digital images were analyzed in MatLab (Mathworks Inc., Natick, MA, USA). The total stomach was outlined in the composite image (summation of all images) with a cursor over the largest anterior gastric image obtained by ingestion of 180 mL. The outline for each 180 mL analysis was individually copied and fitted in the 60 mL image of the same subject. This outline was then subdivided into two regions of interest (ROIs) corresponding to the proximal and distal stomach, according to each method used (Figure 1): ROIs 1: The proximal stomach region was the "reservoir" area seen in all subjects in the first postprandial frames and the line used to divide proximal/distal stomach was drawn immediately below this region[2,7,15]; ROIs 2: The image was divided into two equal areas, designated the proximal and distal stomach, by a mid-length separation in the longitudinal axis of the stomach[1,4,16]; and ROIs 3: The proximal and distal regions of the stomach were separated by a fainter band of radioactivity coinciding with the angula; thus the stomach was arbitrarily divided by drawing a line across the incisura angularis[5,6].

Intragastric distribution of the meal

The time for meal consumption was measured individually. Thus, a value considering 100% retention of the meal was dependent of the activity at the end of the lag phase (the frame before any activity appeared in the small intestine) and immediately after meal completion. Time zero started when the retention was 100%. For each region (total, proximal and distal stomach) activity time curves, expressed as percentages of activity in the total stomach with 100% of meal retention, were obtained.

The intragastric distribution of 60 and 180 mL were assessed from activity time curves derived from each region and considering the three proximal-distal segmentations by calculating the following parameters: (1) initial retention: the percentage of initial activity (%) contained in the total, proximal and distal stomach at time zero; (2) final retention: the percentage of final activity (%) contained in the total, proximal and distal stomach at 18 min; (3) proximal emptying half-time ($T_{1/2}$): expressed as the time (min) when the initial retention in the proximal stomach decreased by 50%; (4) maximal distal content: the highest activity value (%) in the distal stomach at any time point in the study; and (5) gastric emptying of the whole stomach (representing% retention over time) was obtained from time zero to 18 min.

Comparisons of the data for three proximal-distal segmentations were made for both meals employing area under curves (AUC) and statistical moment analysis. The AUC derived from the proximal or distal stomach was expressed as percentage of AUC obtained from total stomach. The statistical moment (minutes) was obtained through the temporal average from the proximal or distal distribution curve, normalized by AUC[17]. This quantification allowed determination of a distribution time that could be associated with the midpoint of the proximal and distal distribution curves.

Contractility

Fast fourier transform (FFT) was employed to analyze phasic contractions in both gastric regions (proximal and distal) and for each type of proximal-distal segmentation. A bi-directional Butterworth band-pass filter with a cutoff frequency at 5-75 mHz (0.3-4.5 cpm) was applied.

Dominant frequencies were expressed as the frequency at which the highest FFT power spectrum was observed in the proximal and distal regions. Values were expressed as power ratios (%), determined by dividing the power of each dominant frequency by the total power (sum of both frequencies), and multiplying the results by 100 for each stomach region in all proximal-distal segmentations[18].

Statistical analysis

Data were expressed as mean ± SE. The hypothesis of a normal data distribution was confirmed using Shapiro-Wilk's test. Data obtained by meals of 60 and 180 mL were compared using Student's *t* test and *P*-values less than 0.05 were considered significant. Comparisons among types of proximal-distal segmentation were analyzed by one-way ANOVA and Tukey's test, with $P < 0.05$ considered significant.

RESULTS

Our data demonstrated a significant effect of meal size and the three proposed proximal-distal segmentations on intragastric meal distribution and gastric contractility. After ingestion, both meals were rapidly dispersed through the whole stomach with a minimal lag phase. There was no difference between the lag-phase for 60 mL (1.9 ± 0.2 min) and for 180 mL (2.3 ± 0.2 min).

The three proposed proximal-distal segmentations (ROIs) could be applied to all volunteers. Figure 2 shows the profile of IDM of the 60 and 180 mL meal over 18 min for the three types of proximal-distal segmentations. A redistribution of food from the proximal to the distal stomach according to employed segmentation was observed.

There was greater proximal retention after 180 mL ingestion compared to 60 mL, which was proportional to the increase in the area of proximal stomach generated by the type of proximal-distal segmentation. ROIs 1 presented a smaller proximal region and a fast redistribution of the meal for the distal area occurred. By contrast, in ROIs 3, the initial retention in the proximal region was greater than in the other proximal-distal segmentations and presented a slow redistribution of the meal for the distal area. ROIs 2 showed an intermediate pattern. Thus, there was a significant difference among the three ROIs segmentations employed ($P < 0.05$).

No difference was found in the percentage of the meal retained in the total stomach after 60 mL (13.3% ± 3.0%) and 180 mL (13.0% ± 2.4%) ingestion over 18 min. Table 1 compares the effect of meal size and proximal-distal segmentation in IDM parameters, reinforcing the relationship between proximal area and parameters of regional gastric emptying.

Table 2 presents the mean area under the curve and the statistical moment for the proximal and distal regions, for each of the three types of segmentation, after ingestion of the test meals. For the smaller meal, there was a significant difference among ROIs, while the increased meal size generated a difference only between ROIs 1 and 3.

Moment calculation demonstrated that the differences between the ROIs were only evident with 60 mL but not with 180 mL meal for the three types of proximal-distal segmentation. Comparison of moments for the distal region for both meals showed no significant differences. The statistical moment obtained in the proximal area was influenced by the type of segmentation adopted (Table 2), representing an option to quantify a time related to intragastric distribution.

An important finding was both dominant frequencies were recorded in the proximal stomach (16.0 ± 1.0 mHz or 1 cpm and 50.0 ± 2.0 mHz or 3 cpm) and in the distal stomach (16.0 ± 1.0 and 50.0 ± 1.0 mHz), independently of gastric segmentation. The dominant frequencies were

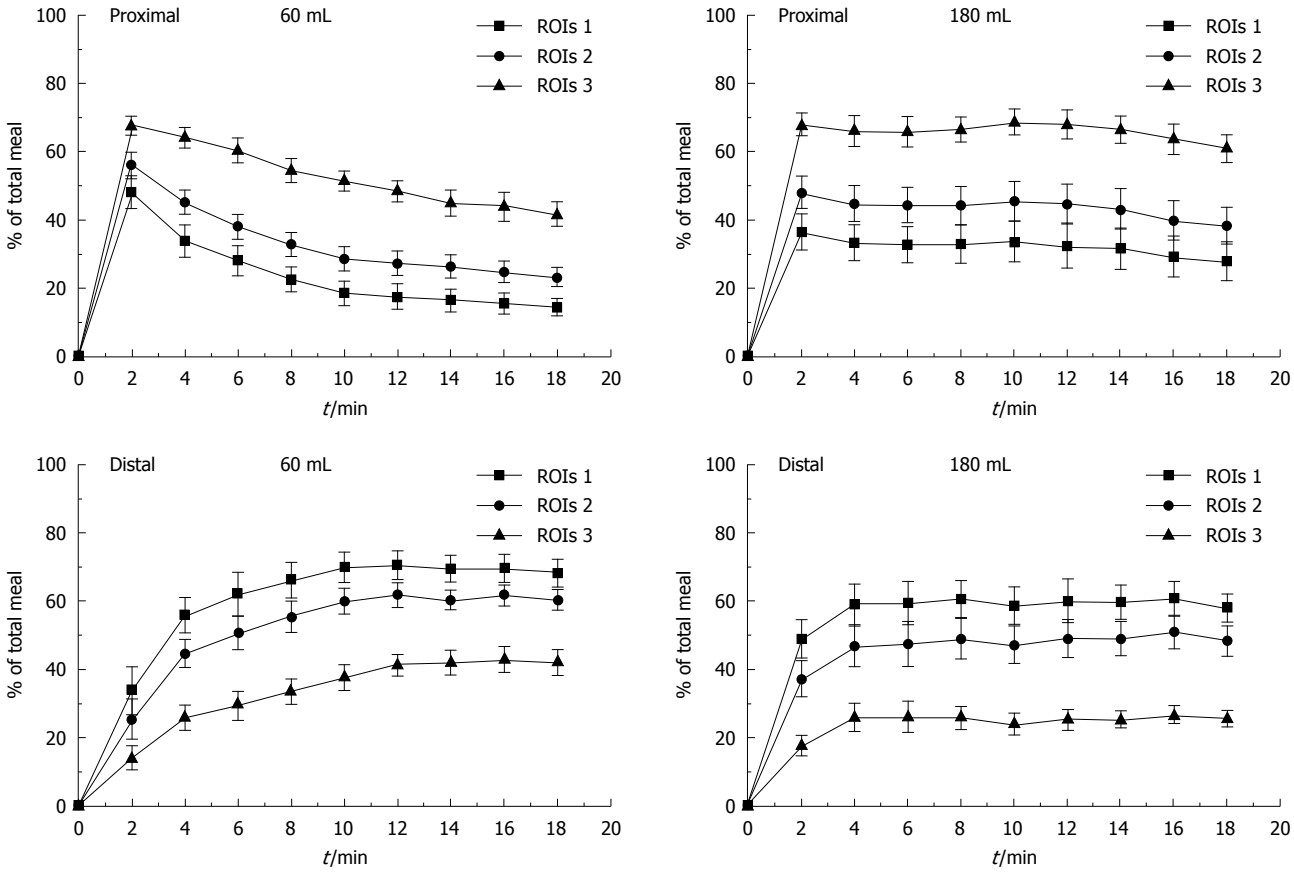

Figure 2 Proximal and distal stomach curves of intragastric distribution according to proximal-distal segmentation ROIs 1, ROIs 2 and ROIs 3 for meals with 60 and 180 mL. Data are expressed as mean ± SE. ROIs: Regions of interest.

Table 1 Effect of meal volume and proximal-distal segmentation in intragastric distribution parameters (mean ± SE)

	60 mL			180 mL		
	ROIs 1	ROIs 2	ROIs 3	ROIs 1	ROIs 2	ROIs 3
Total stomach						
Initial retention	100	100	100	100	100	100
Final retention	82.5 ± 3.8	83.5 ± 3.2	83.8 ± 2.9	84.9 ± 3.3	85.7 ± 3.1	85.5 ± 3.2
Proximal (%)						
Initial retention	39.8 ± 6.8	51.7 ± 6.0	73.2 ± 3.8[a,c]	38.6 ± 6.4	52.3 ± 6.4	75.2 ± 4.3[a,c]
Final retention	14.1 ± 2.5	22.8 ± 3.0	41.6 ± 3.7[a,c]	27.7 ± 5.7[e]	38.3 ± 5.5[g]	60.4 ± 4.2[a,c,i]
T1/2 (min)	4.2 ± 0.6	7.0 ± 0.8	> 18.0	11.0 ± 2.2	> 18.0	> 18.0
Distal (%)						
Initial retention	60.1 ± 6.7	48.1 ± 6.0	26.6 ± 3.8[a,c]	61.4 ± 6.4	47.6 ± 6.4	24.7 ± 4.3[a,c]
Final retention	68.4 ± 4.0	60.7 ± 3.2	42.2 ± 4.0[a,c]	57.1 ± 4.2	47.6 ± 4.5[g]	25.1 ± 2.5[a,c,i]
Maximum content	80.0 ± 4.5	69.0 ± 3.7	48.0 ± 3.6[a,c]	69.0 ± 5.0	56.7 ± 5.7	32.0 ± 3.8[a,c]

[a]$P < 0.05$ *vs* ROIs 1; [c]$P < 0.05$ *vs* ROIs 2; [e]$P < 0.04$ *vs* 60 mL; [g]$P < 0.03$ *vs* 60 mL; [i]$P < 0.003$ *vs* 60 mL. ROIs: Regions of interest.

observed to overlap in the signal of proximal stomach at different time points (Figure 3). Power ratio calculations indicated that there was a rearrangement of the maximum power of each frequency (1 and 3 cpm) according to the segmentation type.

The difference is shown in the power spectrum of these frequencies according to the type gastric segmentation (Figure 4). A difference could be observed in the distal region between 60 and 180 mL: for 180 mL meal there was an increase of the 1 cpm and a decrease of the 3 cpm in ROIs 2 and 3. There were no significant changes for ROIs 1.

DISCUSSION

The results showed that meal distribution in the human stomach differs according to the volume used (60 or 180 mL) and noticeably according to proximal-distal image segmentation used. Dynamic gastric scintigraphy was effective for determining two dominant frequencies (1 and

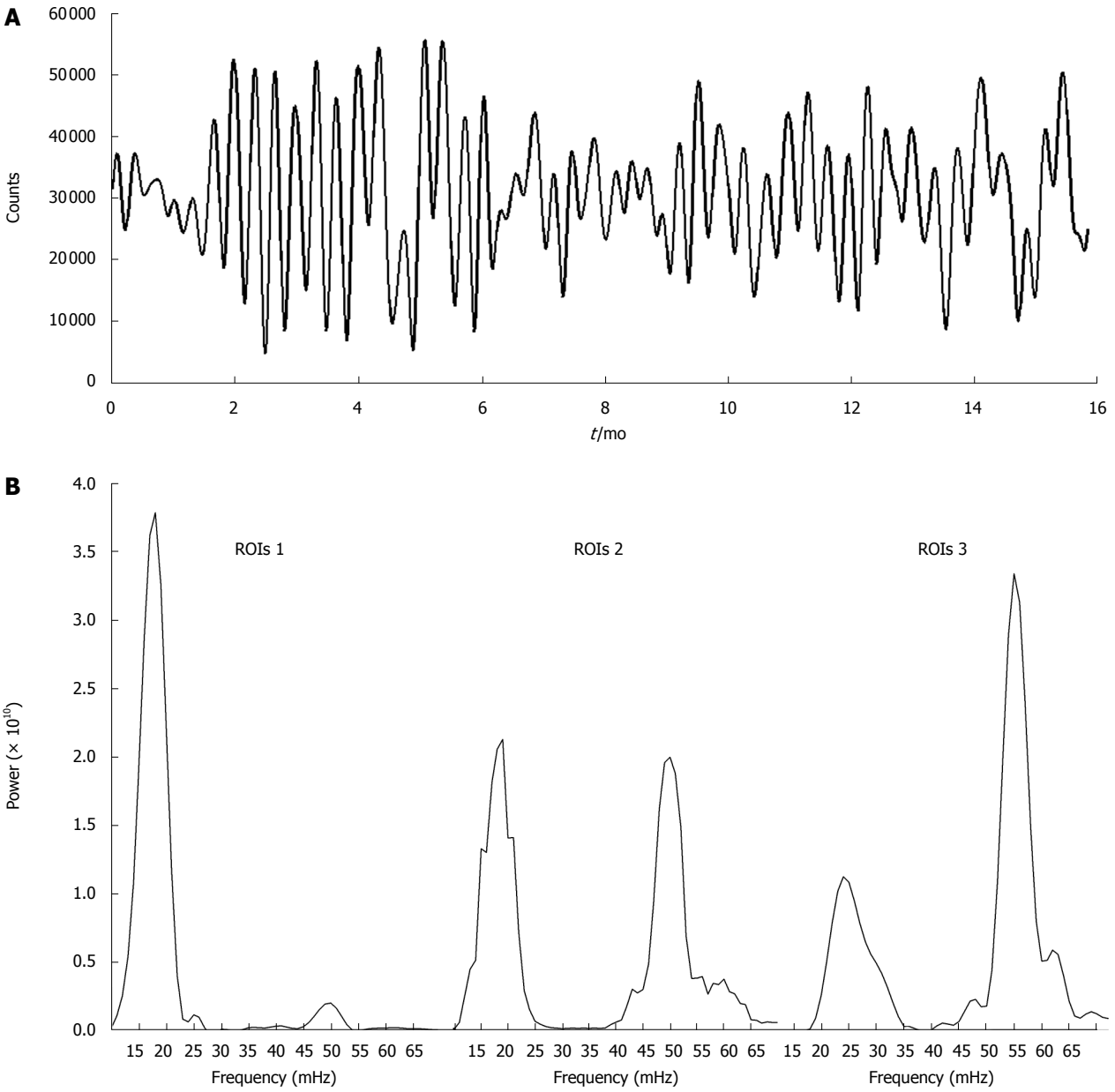

Figure 3 Both dominant frequencies (1 cpm or 17 mHz and 3 cpm or 50 mHz) can be observed overlapping in the signal of the proximal stomach (A), the power ratio of this signal indicated that there was a rearrangement of the maximum power of each of the frequencies (1 and 3 cpm) in agreement with the segmentation type B).

3 cpm), and the magnitude of these contractions in both the proximal and distal stomach.

It has been previously shown that volume and meal size influenced gastric emptying[19,20]; thus, it seems logical to advise patients to decrease the size and increase the frequency of meals during certain conditions, such as pregnancy, gastroesophageal reflux disease, and functional dyspepsia[14]. Our meals were chosen to investigate IDM and motility patterns using semisolid small meals in order to establish a pattern in healthy volunteers. Thereafter, our approach can be applied in patients to verify IDM and gastric motor activity after the recommended consumption of a smaller meal.

The emphasis of most previous studies was on total gastric emptying[19], despite the fact that regional gastric emptying is more frequently abnormal than total gastric emptying[2]. The partitioning of ingested meals between the proximal and distal stomach is related to the genesis of dyspeptic symptoms, such as early satiety, fullness, and nausea[1,4,21]. The meal distribution within the area of the stomach might strongly influence the way in which the stomach is subdivided[4]. Unfortunately, the methods used for defining these two areas are still controversial and poorly defined[15]. Our data demonstrated a direct relationship between gastric compartment size and IDM. The choice of the segmentation technique should consider variations in the stomach shape[16] and the objective of the study. For example, using ROIs 1 it is possible analyze details of the fundic accommodation process' whereas employing ROIs 2 both dominant frequencies

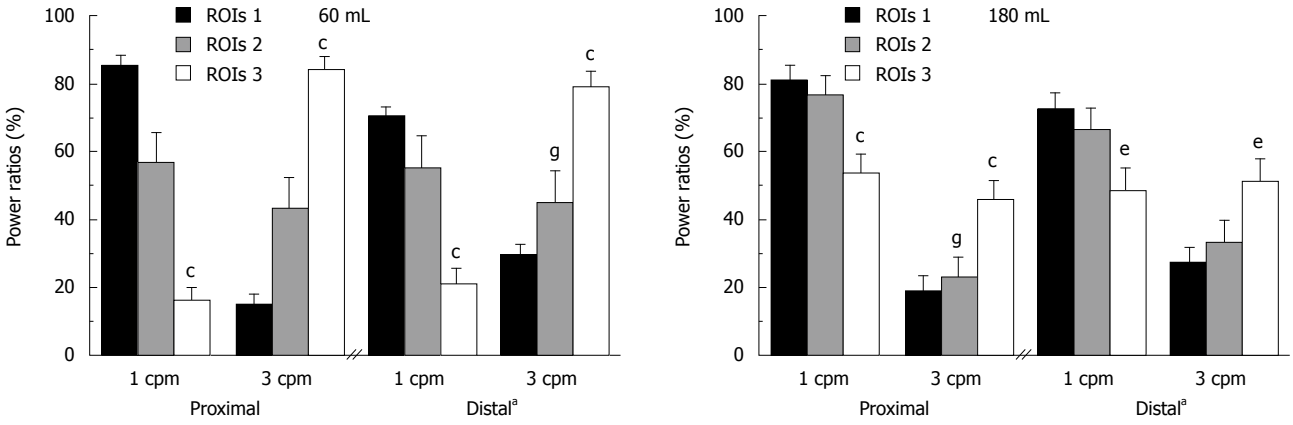

Figure 4 The power ratios showed the presence of both frequencies (1 cpm and 3 cpm) in the proximal and distal stomach, and that their intensities depend on the adopted proximal-distal segmentation and on the meal size ingested. [a]$P < 0.03$ between 60 and 180 mL for both frequencies in distal region, [c]$P < 0.005$ between ROIs 1 and ROIs 3, [e]$P < 0.05$ between ROIs 1 and ROIs 3, [g]$P < 0.05$ between ROIs 2 and ROIs 3. ROIs: Regions of interest.

Table 2 Mean area under the curve and time distribution by statistical moments for proximal and distal regions for the three proximal-distal segmentations, after ingestion of the test meal of 60 and 180 mL (mean ± SE)

	Proximal			Distal		
	ROIs 1	ROIs 2	ROIs 3	ROIs 1	ROIs 2	ROIs 3
AUC (counts.s)						
60 mL	27.2 ± 2.9	31.4 ± 2.5	43.9 ± 1.7[a,c]	72.8 ± 2.9	68.6 ± 2.5	56.1 ± 1.7[a,c]
180 mL	35.3 ± 4.1	39.4 ± 3.2	49.0 ± 3.8[a]	64.7 ± 4.1	60.6 ± 3.2	51.0 ± 3.8[a]
P	< 0.09	< 0.03[1]	< 0.1	< 0.09	< 0.03[1]	< 0.1
Statistical moments (min)						
60 mL	6.9 ± 0.3	7.7 ± 0.2[c]	8.5 ± 0.1[a,c]	10.4 ± 0.3	10.5 ± 0.3	10.7 ± 0.2
180 mL	8.7 ± 0.2	9.0 ± 0.1	9.2 ± 0.1	9.8 ± 0.3	10.0 ± 0.3	10.1 ± 0.3
P	< 0.00001[1]	< 0.00003[1]	< 0.0006[1]	< 0.2	< 0.1	< 0.07

[1]60 mL *vs* 180 mL. [a]$P < 0.05$ *vs* ROIs 1, [c]$P < 0.05$ *vs* ROIs 2, [e]$P < 0.05$ *vs* ROIs 1. ROIs: Regions of interest.

can be equally observed, and ROIs 3 is useful to evaluate antral contractility.

Generally, twenty minutes after the ingestion of a larger meal, a gradual decrease in proximal stomach activity begins, with a corresponding increase in the distal stomach, indicating a redistribution of food from the proximal to the distal stomach[1,4,5]. Hence, our IDM data were obtained during this initial stage, showing that the 60 mL meal quickly began to be redistributed to the distal region; whereas, using 180 mL, there was a slower redistribution from proximal to distal stomach in all segmentations adopted (Figure 2). In the curves obtained from ingestion of 180 mL was impossible calculate $T_{1/2}$ from the proximal stomach during the 18 minutes of recording, except for ROIs 1, reinforcing the retention in the proximal region. In the final retention values, there was a significant difference between three ROIs segmentation and both meals ingested (Table 1).

To quantify the IDM, we used the statistical moments, which have previously been utilized only in pharmaceutical approaches[17], and the traditional AUC. The statistical moments and AUC provide complementary information about the data observed. Both quantification methods showed that proximal-distal segmentations have more effect for the 60 mL meal than for 180 mL. However, there was no difference in the statistical moment between ROIs in the distal curve profile, whereas there was a significant difference in AUC. Distal accumulation time was defined previously by our group as the time elapsing from the meal ingestion until the activity reached 99% of the maximum value in the sigmoidal tracing over the distal stomach curves[12]. The distribution time obtained by the statistical moment was very close to the distal accumulation time, but can be employed for any kind of curve, including proximal stomach curves.

It is important to emphasize that partitioning of ingested meals between the proximal and distal stomach is related to gastric accommodation. Abnormal IDM might be a consequence of disturbed proximal stomach accommodation[4] in a considerable subset of patients with functional dyspepsia and might have a role in symptom production, such as early satiety and weight loss[6]. Studies of dyspepsia have shown a preferential accumulation in the distal stomach, suggesting defective postprandial relation of the proximal stomach; however, it is difficult to draw general conclusions because each study employed a different proximal-distal partition. It would be interesting to provide data on the relationship between meal size, proximal-distal segmentation, IDM, and accommodation in this patient group. Information on the accommodation

process will have clinical value, especially for studying patients with dyspepsia and normal gastric emptying, and it may contribute directly to improved medical therapy[22].

Fast Fourier transformation of our scintigraphic recordings defined two dominant frequencies in the distal as well as the proximal stomach, in all volunteers (Figure 3). Meal size did not affect the dominant frequencies of the contractions or their power, but there was a large difference in the power spectra of these frequencies, based on image segmentation. The power ratio of the proximal signal was rearranged for the maximum power of each frequency (1 and 3 cpm), according to segmentation type. Hence, there was a power gradient from ROIs 1 to ROIs 3, where 1 cpm decreases and 3 cpm increases in the human proximal stomach (Figures 3 and 4). This motor activity around 1 cpm on the proximal stomach[9,10,23] has not been extensively documented in humans, although it has been correlated with functional dyspepsia[9], mainly due to methodological issues, such as differences in barostat systems[24] and/or filter parameters employed in data analysis[12].

In summary, the results of research and/or diagnosis can be deeply influenced by the proximal-distal segmentation method adopted. Two dominant frequencies (1 and 3 cpm) can be simultaneously registered in the proximal and distal stomach, but the proximal-distal segmentation should be considered carefully to analyze their power spectra. The protocol developed in this study can be applied in patients with several disorders, with the advantages of simultaneous evaluation of IDM and gastric contractions.

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COMMENTS

Background

The stomach is composed of two distinct functional regions. The distal stomach is capable of generating 3 vigorous contractions per minute (cpm), which cause reduction in size of ingested particles and subsequent emptying. The proximal stomach is primarily concerned with storage of the ingested food, with a slight contraction activity around 1 cpm. During a meal, the stomach continuously adapts its size to the content by gradually relaxing its musculature, performing the so-called accommodation to distension. Intragastric distribution of the meal (IDM) between the proximal and distal stomach is related to the accommodation process, and is useful for defining abnormal physiology and for explaining certain functional disease symptoms.

Research frontiers

Proximal-distal segmentation approaches employed to divide the stomach into proximal and distal regions remain a challenge in the study of IDM. Unfortunately, the methods used for defining these two areas are still controversial and poorly defined. Validation is necessary before incorporating these methods into clinical practice. Two dominant frequencies (1 and 3 cpm) can be registered simultaneously in the proximal and distal stomach, but the proximal-distal segmentation should be considered carefully to analyze their power spectra. The motor activity around 1 cpm on the proximal stomach has been correlated with functional dyspepsia. In this context, the frequency of contraction can be explored to elucidate certain disease patterns.

Innovations and breakthroughs

Scintigraphy already is the gold standard for measuring gastric emptying and offers the advantage of completely characterizing the complex physiology of IDM between stomach regions. New studies can be exploited to refine and extend its use in clinical practice. The functions of the stomach regions can vary according to the nutrient content and there is a clinical recommendation for consumption of smaller and more frequent meals to avoid postprandial symptoms in patients with common gastrointestinal disorders. However, there is little information about the effects of meal size on intragastric distribution, especially for semisolid small meals. In the area of functional disorders research, considerable effort is being expended on how to convert basic knowledge into benefits for patients' treatment. Therefore, in the present study we compared three kinds of segmentation in normal volunteers and showed that segmentation is remarkably important in the evaluation of IDM and gastric motility. This observation is particularly relevant when assessing patients.

Applications

Abnormal IDM might be a consequence of disturbed proximal stomach accommodation in a considerable subset of patients with functional dyspepsia and might have a role in symptom production, such as early satiety and weight loss. Studies of dyspepsia have shown a preferential accumulation in the distal stomach, suggesting defective postprandial accommodation in the proximal stomach; however, it is difficult to draw general conclusions, because each study employed a different proximal-distal partition. Thus, providing data on the relationship between meal size, proximal-distal segmentation, IDM, and accommodation in this patient group is very important. Information about the accommodation process will have clinical value, especially in the study of patients with dyspepsia and normal gastric emptying, and might directly influence medical therapy.

Terminology

IDM represents the distribution of gastric contents between the proximal and distal stomach during gastric emptying. Dyspepsia is a medical condition characterized by chronic or recurrent pain in the upper abdomen, bloating, and fullness.

Peer review

The authors evaluated the effects of meal size and three segmentations on intragastric distribution of meal and gastric motility by scintigraphy. This paper is an interesting report.

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BRIEF ARTICLE

PPIs are not associated with a lower incidence of portal-hypertension-related bleeding in cirrhosis

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Abstract

AIM: To determine if proton pump inhibitor use in cirrhotic patients with endoscopic findings of portal hypertension is associated with a lower frequency of gastrointestinal bleeding.

METHODS: Patients with cirrhosis and endoscopic findings related to portal hypertension, receiving or not receiving proton pump inhibitor (PPI) therapy, were included retrospectively. We assigned patients to two groups: group 1 patients underwent PPI therapy and group 2 patients did not undergo PPI therapy.

RESULTS: One hundred and five patients with a median age of 58 (26-87) years were included, 57 (54.3%) of which were women. Esophageal varices were found in 82 (78%) patients, portal hypertensive gastropathy in 72 (68.6%) patients, and gastric varices in 15 (14.3%) patients. PPI therapy was used in 45.5% of patients (n = 48). Seventeen (16.1%) patients presented with upper gastrointestinal bleeding; in 14/17 (82.3%) patients, bleeding was secondary to esophageal varices, and in 3/17 patients bleeding was attributed to portal hypertensive gastropathy. Bleeding related to portal hypertension according to PPI therapy occurred in 18.7% (n = 9) of group 1 and in 14% (n = 8) of group 2 (odds ratio: 0.83, 95% confidence interval: 0.5-1.3, P = 0.51).

CONCLUSION: Portal hypertension bleeding is not associated with PPI use. These findings do not support the prescription of PPIs in patients with chronic liver disease with no currently accepted indication.

Key words: Drug prescription; Liver cirrhosis; Portal hypertension; Proton pump inhibitors; Upper gastrointestinal bleeding

Peer reviewer: Dr. Lisardo Boscá, Professor, Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM), Arturo Duperier 4, 28029 Madrid, Spain.

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INTRODUCTION

Since their first clinical use, proton pump inhibitors (PPIs)

have provided benefits in the management of gastrointestinal diseases. This class of drugs is clearly indicated for the treatment of peptic ulcer disease, gastroesophageal reflux disease (GERD), and nonvariceal upper gastrointestinal bleeding, and for prophylaxis in selected users of nonsteroidal anti-inflammatory drugs (NSAIDs)[1,2]. Unfortunately, the unnecessary prescription of PPIs has become an important problem, which increases economic costs in daily clinical practice[3,4]. According to previous studies in the clinical context, only 12.3% of cirrhotic patients have an appropriate indication for the prescription of these drugs[5]. Congestive gastropathy and esophageal and gastric varices are risk factors for the inappropriate use of PPIs[5].

Few studies, other than pharmacological studies, have investigated the safety and utility of PPIs in cirrhotic populations[6-9]. There are reports of possible hepatotoxicity associated with the use of PPIs in patients with chronic liver disease (CLD)[9], but there have been no clinical or experimental trials on the adverse effects of PPIs in the treatment of acute or chronic complications in patients with cirrhosis and portal hypertension (PH). Some data on the possible use of PPIs for the long-term prophylaxis of variceal bleeding exist[10], and a recent controlled trial by Zhoe *et al*[11] compared the efficacy of octreotide, vasopressin, and omeprazole for controlling acute bleeding associated with portal hypertension gastropathy. However, more clinical evidence is required. The use of this class of drugs in cirrhotic patients seems more habit-related than evidence-based, ultimately leading to an increase in health costs.

In patients with cirrhosis and PH, upper gastrointestinal bleeding has an annual frequency of 25%-35%, and 80%-90% is of variceal origin. The mortality related to variceal bleeding is about 30% per episode, and is recurrent in 70% of patients after the first year[12-15].

Considering the current paradigm of evidence-based medicine, the use of PPIs in patients with cirrhosis and endoscopic findings of PH is based only on expert opinion, with insufficient evidence to justify the use of these drugs as prophylaxis for variceal bleeding. The aim of this study was to determine whether the use of PPIs in patients with cirrhosis and endoscopic findings of PH (esophageal or gastric varices, or portal hypertensive gastropathy) is associated with a reduction in the frequency of gastrointestinal bleeding secondary to PH.

MATERIALS AND METHODS

We conducted a retrospective, observational, longitudinal, comparative study of outpatients with CLD and endoscopic evidence of PH, receiving or not receiving treatment with PPIs, between December 1, 2004 and January 1, 2006. The endoscopic data considered for PH were esophageal varices, gastric varices, and portal hypertensive gastropathy. The sample comprised a series of consecutive patients with clinical, biochemical, endoscopic, radiological, and/or histological signs of cirrhosis and PH who attended our gastroenterology and liver clinic. We included all patients over 18 years of age who had been reviewed on at least two visits over the course of one year during the period of the study. All patients with incomplete electronic or paper charts, with no confirmatory endoscopic study at the time of the bleeding episode, were excluded from the study. These patients formed a subset of patients included in our previous work[5]. Reasons for exclusion from the present study were absence of endoscopic evidence of PH (n = 80), and no previous endoscopy (n = 28).

The primary demographic and medical variables were age, sex, etiology of CLD, diagnosis of hepatocellular carcinoma, liver function tests, presence of ascites, encephalopathy, the model end-stage liver disease (MELD) score, and previous use of NSAIDs (at least five times per week during the last six months), cyclooxygenase-2 inhibitors, corticosteroids, anticoagulants, and aspirin. Any hospital stay associated with portal hypertensive bleeding was also recorded.

An endoscopic procedure was performed in all patients as an initial approach. Any patient with first endoscopy at the time of an episode of active bleeding was included. The primary endpoint of our study was the presence of portal hypertensive bleeding. We defined bleeding related to PH as any bleeding episode secondary to the rupture or erosion of esophageal or gastric varices and/or portal hypertensive gastropathy, manifested clinically as melena or hematemesis. All patients with suspected variceal bleeding during the period of the study were required to have an endoscopic procedure in the first 24 h after presentation. A regular diagnostic endoscope was initially used (GIF-100, GIF-130, GIF-140, or GIF-160; Olympus, Japan). The presence of esophageal or gastric varices, portal hypertensive gastropathy, red signs, and the size of the varices were recorded according to the Baveno Ⅳ consensus[16]. Other variables assessed included nonliver-related findings such as esophagitis, hiatal hernia, erosive gastritis, and duodenal or gastric ulcer.

The use of PPIs and other medical prescriptions within the six months preceding the study were identified in the patients' records. We defined PPI users as those patients with cirrhosis who had taken 20 mg of omeprazole (or an equivalent dose of any other PPI) for at least eight weeks before the episode of portal hypertensive bleeding or initial evaluation (first considered visit). Confirmation of the patients' compliance with the PPI treatment was based on chart records. A diagnosis of GERD was made according to the definition: "a condition that develops when the reflux of stomach contents causes troublesome symptoms and/or complications"[17]. Troublesome symptoms were defined by the patient as affecting his/her quality of life. The symptoms considered were heartburn, regurgitation, reflux-related chest pain, extraesophageal syndromes of GERD (laryngitis, cough, asthma) confirmed by their resolution with PPI therapy, pH monitoring, or endoscopic evidence of esophagitis, according to the Los Angeles classification (grades B, C, or D)[17].

Statistical analysis

The results are expressed as distributions, absolute frequencies, relative frequencies, medians and ranges, or

means ± SD. For comparison, patients were classified into two groups: patients who used PPIs and patients who did not use PPIs. The quantitative data were compared using the Student's *t*-test for variables with a normal distribution, and the Mann-Whitney *U* test for other variables. Differences between the proportions of categorical data were evaluated with Fisher's exact test when the number of expected subjects was less than five and otherwise with the χ^2 test. A multivariate logistic regression model was used to assess the independent association between PPI use and bleeding related to PH. A *P* value of < 0.05 was considered statistically significant.

Sample size calculation

According to data published by Hajime *et al*[10] the frequencies of variceal bleeding in patients with and without PPI use were 10% and 52.4%, respectively (a difference of 42%). According to these data, to detect a difference of at least 42%, we required at least 25 patients for each group (group 1, patients with cirrhosis and PPI use; and group 2, patients with cirrhosis and no PPI use). All statistical analyses were conducted with SPSS statistical software (v. 12.0; SPSS Inc., Chicago, IL, USA).

RESULTS

We initially evaluated 135 patients. Thirty patients were excluded because of incomplete data, therefore, a total of 105 patients were included in the study. The characteristics of the included patients are shown in Table 1. The most frequent endoscopic finding was esophageal varices in 82 (78%) patients, 16 (19.5%) of whom were recorded as having large varices and/or red signs. Portal hypertensive gastropathy was found in 72 patients (68.6%) and gastric varices in 15 patients (14.3%). Of those patients with gastric varices, 13/15 (86.6%) also had esophageal varices. Other findings not related to CLD were erosive gastropathy in 14 patients (13.3%), hiatal hernia in eight patients (7.6%), duodenal ulcer (Forrest Ⅲ) in three patients (2.9%), and gastric ulcer (Forrest Ⅲ) in three patients (2.9%). Other comorbidities are shown in Table 1. There was a tendency [odds ratio (OR): 1.3, 95% confidence interval (CI): 0.72-2.6, $P = 0.2$] to non-portal hypertension-related bleeding episodes ($n = 20$; erosive gastropathy, duodenal ulcer, and gastric ulcer) in patients not using PPIs.

Forty-eight (45.5%) patients underwent PPI therapy. Most of these patients used omeprazole, although 10 used pantoprazole. During the period of evaluation, 16.1% ($n = 17$) presented with upper gastrointestinal bleeding related to PH, and in 82.3% of these patients ($n = 14$), this bleeding was secondary to esophageal varices, whereas in three patients it was attributable to portal hypertensive gastropathy. We recorded no episodes of bleeding secondary to gastric varices. When we analyzed the presence of variceal bleeding in patients classified according to their pattern of PPI use (group 1, patients using PPI, $n = 48$; and group 2, patients not using PPI, $n = 57$), the frequency was 18.7% ($n = 9$) in group 1 and 14% ($n = 8$) in group 2 (OR: 0.83, 95% CI: 0.5-1.3, $P = 0.51$). When we evaluated only those patients with upper gastrointestinal bleeding secondary to esophageal varices, we observed frequencies of 12.5% in group 1 and 14% in group 2 (OR: 1.07, 95% CI: 0.56-2.0, $P = 0.81$). A comparison of the characteristics of patients using PPIs and those not using PPIs is shown in Table 1.

Table 1 Characteristics of the patients included in the study classified by proton pump inhibitor use (mean ± SD) *n* (%)

Variable	Patients using PPIs ($n = 48$)	Patients not using PPIs ($n = 57$)	*P* value
Age (yr)	56.1 ± 13.8	57 ± 12.4	0.71
MELD	12.8 ± 6.3	11.5 ± 5.4	0.25
CPT	8.3 ± 1.8	7.2 ± 2.2	0.55
Albumin (g/L)	28 ± 0.6	32 ± 1.5	0.16
Total bilirubin (mg/L)	27 ± 3.7	24 ± 3.3	0.62
ALT (UI/L)	47.2 ± 22.6	50 ± 40.5	0.66
Alkaline phosphatase (UI/L)	161.2 ± 92.2	132.9 ± 63.8	0.06
BMI	26.5 ± 4.4	25.9 ± 3.7	0.46
Sex, male	19 (40)	29 (51)	0.24
Etiology			
Viral hepatitis C	25 (52)	25 (44)	0.44
Alcohol	12 (25)	12 (21)	0.56
Cryptogenic	5 (10)	10 (18)	0.28
Autoimmune hepatitis	2 (4)	8 (14)	0.22
Other	4 (8)	2 (4)	0.26
Child-pugh-turcotte			
A	19 (40)	31 (54)	0.2
B	22 (46)	17 (30)	0.3
C	7 (15)	9 (16)	0.4
GERD	7 (15)	5 (9)	0.7
Gastric/esophageal varices	44 (92)	40 (70)	0.006
Large	6 (13)	3 (5)	0.1
Red signs	4 (8)	3 (5)	0.35
Responders to β-adrenergic blocker	13 (27)	11 (19)	0.34
NSAID	4 (8)	0 (0)	0.04
Antiplatelet agents use	7 (15)	5 (9)	0.1
Oral anticoagulation	1 (2)	1 (2)	0.9
Steroid use	3 (7)	2 (4)	0.37
Comorbidities			
Diabetes mellitus	19 (40)	28 (49)	0.43
Hypertension	9 (19)	14 (25)	0.63
High-level triglycerides	3 (6)	10 (18)	0.13

CPT: Child-pugh-turcotte class; GERD: Gastroesophageal reflux disease; MELD: Model for end stage liver disease; PPIs: Proton pump inhibitors; ALT: Alanine aminotransferase; NSAID: Non-Steroidal anti-inflammatory drugs; BMI: Body mass index (calculated as patient body weight divided by the square of their height expressed in kg/m^2).

The overall prevalence of GERD was 11.4% ($n = 12$), corresponding to 14.5% of group 1 ($n = 7/48$). Only seven (57.1%) patients with GERD received PPIs. Of the total number of patients with portal hypertensive bleeding, 11.7% ($n = 2/17$) had GERD. The presence of GERD was not statistically significantly associated with the presence of upper gastrointestinal bleeding (OR: 0.53, 95% CI: 0.15-1.8, $P = 0.31$). Univariate and multivariate analyses of the variables associated with gastrointestinal bleeding secondary to PH are shown in Table 2.

DISCUSSION

In this study, we observed that in patients with CLD and endoscopic evidence of PH, the presence of gastrointes-

Table 2 Univariate and multivariate analyses of risk factors associated with portal-hypertension related bleeding in cirrhotic patients

	B Coefficient	Standard error	Wald χ^2	OR (95% CI)	*P* value
Univariate					
Age ⩾ 60	-	-	-	1.1 (0.37-3.5)	1
Sex, male	-	-	-	1.7 (0.5-5.2)	0.39
CPT C	-	-	-	1.6 (0.40-6.6)	0.36
GERD	-	-	-	0.53 (0.15-1.8)	0.31
MELD > 15	-	-	-	1.2 (0.35-4.4)	0.47
PPI use	-	-	-	0.83 (0.5-1.3)	0.51
LEV	-	-	-	12 (3-123)	< 0.001
Red signs	-	-	-	10 (2-58)	< 0.001
NSAID	-	-	-	0.86	0.55
Multivariate					
LEV	23.7	15.1	0	10 (4-110)	< 0.001
Red signs	22.0	14.2	0	9 (4-102)	< 0.001

CPT C: Child-pugh-turcotte class C; GERD: Gastroesophageal reflux disease; MELD: Model for end stage liver disease; PPI: Proton pump inhibitor; LEV: Large Esophageal varices; NSAID: Non-steroidal anti-inflammatory drugs; OR: Odds ratio; CI: Confidence interval.

tinal bleeding secondary to variceal or portal hypertensive gastropathy was not associated with the use of PPIs. This is a very important finding because it has been reported that the presence of PH on endoscopy is associated with an unacceptable, and according to our data, unnecessary prescription of PPIs in patients with cirrhosis[5,18].

Soon after the introduction of PPIs into clinical practice, these drugs demonstrated their effectiveness in several gastrointestinal diseases. However, the overuse of this class of drugs has important economic implications. In patients with cirrhosis, many factors influence the appropriate prescription of PPIs[5,18]. It was observed in previous studies that patients in the early stages of Child-Pugh-Turcotte classification, and with a low MELD score, were more likely to be prescribed PPIs appropriately than those in the more advanced stages of the disease or with endoscopic findings of PH[5,18]. According to the scarce evidence available regarding the use of these drugs in the clinical context, it seems that physicians tend to consider the use of PPIs in cirrhotic patients as possibly beneficial for variceal bleeding. It has also been postulated in the past that gastroesophageal reflux may contribute to esophagitis and variceal bleeding in patients with CLD[19]. In fact, there are few data on the use of PPIs in these patients, and these data are predominantly related to the pharmacological properties of the drugs[20]. The findings of our study are consistent with those of other studies, which have reported that patients with PH, and especially those with portal hypertensive gastropathy, display increased bicarbonate production and an elevated gastric pH. The increased circulatory rate in these patients, the high gastric pH level, and the increased prevalence of hypochlorhydria are factors associated with lower pepsin activity[21-25].

The main limitation of our study is its retrospective design. However, data concerning the association of portal hypertensive bleeding with the use of PPIs are scarce and are based on only one study, published as an abstract[10]. There is an absence of data from randomized trials, thus, prospective studies are still required to develop more reliable recommendations regarding the use of PPIs in this context. The diagnosis of PH in this study was based on esophageal varices, gastric varices, and hypertensive gastropathy, therefore, it is possible that some patients with a hepatic venous pressure gradient above 12 mmHg were overlooked. However, our study focused on patients with endoscopic findings related to PH.

In conclusion, our data support the hypothesis that the use of PPIs is not associated with upper gastrointestinal bleeding related to PH in cirrhotic patients. Therefore, these findings do not support the use of PPIs in patients with CLD and endoscopic evidence of PH without a currently accepted indication.

COMMENTS

Background

Gastrointestinal bleeding secondary to portal hypertension is a major complication in patients with cirrhosis, and proton pump inhibitors are frequently used to prevent it. These drugs have provided benefits in the management of many gastrointestinal disorders; unfortunately, the unnecessary prescription of these drugs has become an important problem, which increases costs in daily practice. Considering the current paradigm of evidence-based medicine, their use in patients with cirrhosis and portal hypertension is based only on expert opinion, with insufficient evidence to justify the use of these drugs as prophylaxes.

Research frontiers

Proton pump inhibitors are widely used among patients with chronic liver disease and endoscopic findings of portal hypertension with the aim of preventing bleeding, however, there is no appropriate evidence to support their use for this condition. In this study, the authors demonstrate that the use of proton pump inhibitors is not associated with a lower frequency of gastrointestinal bleeding in cirrhotic patients.

Innovations and breakthroughs

In this study, the authors observed that in patients with chronic liver disease and endoscopic evidence of portal hypertension, the presence of gastrointestinal bleeding secondary to variceal or portal hypertensive gastropathy was not associated with the use of proton pump inhibitors. This is a very important finding because it has been reported that the presence of portal hypertension on endoscopy is associated with an unacceptable, and according to the data, unnecessary prescription of proton pump inhibitors in patients with cirrhosis.

Applications

This study provided evidence on the use of proton pump inhibitors in patients with chronic liver disease and endoscopic findings of portal hypertension, and does not support their use without a currently accepted indication for their prescription in this group of patients.

Terminology

Proton pump inhibitors are a class of drugs that reduce the secretion of HCl in the stomach, consequently increasing gastric pH. The current principal accepted indications for these drugs include peptic ulcer disease and gastroesophageal reflux disease. Esophageal varices and hypertensive gastropathy are referred to as endoscopic findings of portal hypertension. Portal hypertension is the main cause of gastrointestinal bleeding in patients with cirrhosis, and its prevention is very important in clinical practice.

Peer review

This is a clear cut-off of the question of non-steroidal anti-inflammatory drugs with/without proton-pump inhibitors administration dilemma. The clinical problem is well addressed and presented, and the authors provide a rationale for their conclusions.

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BRIEF ARTICLE

Metabolomics of gastric cancer metastasis detected by gas chromatography and mass spectrometry

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Abstract

AIM: To elucidate the underlying mechanisms of metastasis and to identify the metabolomic markers of gastric cancer metastasis.

METHODS: Gastric tumors from metastatic and non-metastatic groups were used in this study. Metabolites and different metabolic patterns were analyzed by gas chromatography, mass spectrometry and principal components analysis (PCA), respectively. Differentiation performance was validated by the area under the curve (AUC) of receiver operating characteristic curves.

RESULTS: Twenty-nine metabolites were differentially expressed in animal models of human gastric cancer. Of the 29 metabolites, 20 were up-regulated and 9 were down-regulated in metastasis group compared to non-metastasis group. PCA models from the metabolite profiles could differentiate the metastatic from the non-metastatic specimens with an AUC value of 1.0. These metabolites were mainly involved in several metabolic pathways, including glycolysis (lactic acid, alaline), serine metabolism (serine, phosphoserine), proline metabolism (proline), glutamic acid metabolism, tricarboxylic acid cycle (succinate, malic acid), nucleotide metabolism (pyrimidine), fatty acid metabolism (docosanoic acid, and octadecanoic acid), and methylation(glycine). The serine and proline metabolisms were highlighted during the progression of metastasis.

CONCLUSION: Proline and serine metabolisms play an important role in metastasis. The metabolic profiling of tumor tissue can provide new biomarkers for the treatment of gastric cancer metastasis.

Key words: Gastric cancer; Metastasis; Metabolite; Metabolomics; Gas chromatography and mass spectrometry

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INTRODUCTION

Gastric cancer is one of the most common malignancies and the second cause of cancer-related death worldwide and in most Asian countries, such as China[1,2]. So far, surgical resection remains the only curative treatment option[3]. However, because of its asymptomatic properties,

most patients are frequently misdiagnosed until local and distant metastases occur, leading to a poor prognosis of gastric cancer patients with a 5-year survival rate of less than 30%. Most gastric cancer-related deaths occur as a result of metastasis. Metastatic recurrence is the main obstacle to the improvement of therapy for gastric cancer. No effective treatment modality is available for this deadly disease at present. Similarly, almost no prophylactic therapies can block dissemination of gastric cancer cells and prevent its metastasis. Currently, histological staging of gastric cancer is mainly based on the depth of its invasion and metastasis, both of which are considered the most important indicators of recurrence and prognosis of gastric cancer after curative resection. It has been shown that some gene candidates, such as cell adhesion molecules, are involved in the process of gastric cancer metastasis[3-5]. However, no routine molecule markers for predicting gastric cancer metastasis and prognosis are available because of their high variability in expression levels.

The prognosis of patients with advanced gastric cancer remains very poor because the molecular mechanism underlying its metastasis is not fully understood[3,5]. Gastric cancer metastasis, which is a complex and multistep process, involves release, migration and penetration of its cells through the vessel walls, arrest of its cells in microcirculation of distant organs and their subsequent migration and growth at the metastatic sites. To get a better insight into the mechanism of such a process, metastasis and non-metastasis animal models of gastric cancer were established using the human gastric cancer cell line SGC-7901. Because of the same genetic backgrounds, the animal is a suitable comparative system for studying the molecular changes in gastric cancer metastasis. At present, most investigations are focused on the identification of altered genes and proteins that play an important role in gastric cancer progression[6-9]. However, only a few reports are available on the identification of key metabolites characterizing gastric cancer metastasis[6,10,11]. Metabolomics, an omic science in systems biology, is the comprehensive and simultaneous profiling of metabolic changes occurring in living systems in response to genetic, environmental, or lifestyle factors[11]. The missing link between genotype and phenotype can be established, which may provide information about gastric cancer that is complementary to genomics and proteomics analysis, thus improving our understanding of the pathogenic mechanisms and metabolic phenotype of gastric cancer. It has been recently shown that metabolomic method has great potentials in identifying the new diagnostic markers and therapeutic targets for different cancers, such as breast, prostate, pancreatic, liver, colon and gastric cancers[12-18], suggesting that metabolic alterations play a role in the biology of cancer. A more recent metabolomic analysis showed that increased sarcosine synthesis is an important metabolic change during prostate cancer progression[6]. However, to our knowledge, there is no metabolomic study on metastasis of gastric cancer[6,10].

In this study, metabolomic difference in metastasis and non-metastasis models of gastric cancer was detected by gas chromatography (GC) and mass spectrometry (MS), respectively, in accordance with our hypothesis that there were metabolite clusters associated with metastasis of gastric cancer.

MATERIALS AND METHODS

Chemicals

Tetrahydrofuran (THF), N-methyl-N-t-butyldimethylsilyltrifluoro-acetamide (MBDSTFA) and chromatographic pure were purchased from Sigma Chemical Co. (St Louis, MO, USA). Pyridine, sodium hydroxide, chloroform, anhydrous ethanol, and anhydrous sodium sulfate, purchased from China National Pharmaceutical Group Corporation (Shanghai, China), were of analytical grade. Vacuum dryer was the product of Shanghai NOTED Technologies (China). Distilled water was obtained from the Milli-Q System (Millipore, MA, USA).

Animals

Male mice with severe combined immune deficiency (SCID) at the age of 6 wk, weighing 20-25 g, were obtained from Shanghai Experimental Animal Center, Chinese Academy of Sciences (China). The mice were housed under specific pathogen-free conditions with free access to food and drinking water. Experiment was performed in accordance with the Chinese National Guidelines for the Care and Use of Laboratory Animals and the relative ethical regulations of our university.

Animal treatment

Twenty-two SCID mice were randomly divided into metastasis group (n = 8), non-metastasis group (n = 8), and control group (n = 6). An animal model of metastasis was induced by orthotopic implantation of histologically intact tissue of human gastric carcinoma as previously described[19] with some minor modifications. Human gastric cancer SGC-7901 (Shanghai Cancer Institute), a poorly-differentiated adenocarcinoma line, was originally derived from a primary tumor and maintained by passage in subcutis of nude mice. Tumors were removed aseptically with necrotic tissues cleaved away. The adjacent healthy tissues were cut into pieces (about 3 mm × 4 mm) in Hank's balanced salt solution, weighed and adjusted to 100 mg. After the SCID mice were anesthetized with 4.3% trichloraldehyde hydrate, an incision was made through the left upper abdominal pararectal line of mice in metastasis group with the peritoneal cavity carefully exposed. A part of the serosal membrane in middle of the greater curvature of stomach was mechanically injured using scissors. One hundred mg of tumor pieces was fixed at each injured site on the serosal membrane surface. After the stomach was returned to the peritoneal cavity, the abdominal wall and skin were closed. After the SCID mice were anesthetized with 4.3% trichloraldehyde hydrate, an incision was made in the left oxter of mice in non-metastasis group. Then, 100 mg of tumor tissue pieces was implanted into the subcutis (ectopic implantation) of mice. Meanwhile, the mice in non-metastasis group underwent the same ortho-

topic operation as those in metastasis group but with no tumor implantation into the gastric wall. The mice that underwent the same procedure with no tumor implantation served as a control group. All animals tolerated the surgical procedure well with no anesthesia-related death.

Sample collection and pathological examination

After anesthesia, all mice were sacrificed and subjected to autopsy. Tumors growing at the orthotopic or subcutaneous sites were harvested. Half of each tumor was snap-frozen at -80℃, while the other half was fixed in 4% formalin and embedded in paraffin. Tissues were collected from lymph nodes and all organs, fixed in 4% formalin, and processed for routine paraffin embedding after careful macroscopic examination. The tissue was cut into 4 μm-thick sections which were stained with hematoxylin and eosin, and evaluated histologically for metastasis of lymph nodes, liver or other organs by microscopy.

Sample pretreatment and derivation

One mL stilled water and one mL anhydrous ethanol were added to each 100 mg tissue sample. Gastric carcinoma tissue samples were ground with stilled water and anhydrous ethanol (1:1, v/v). The mixture was ultrasonicated at 4℃ for 30 min and vortexed for 2 min. The tissue sample (0.1 g/mL) was centrifuged at 18 000 × *g* (10 000 r/min) for 10 min. The aqueous layer was adjusted to pH 7.8 with 0.1 mol/L potassium phosphate buffer solution, and 1.5 mL supernatant was obtained from each sample. The collected supernatant (500 μL) was evaporated to dryness at 50℃ for 24 h in a vacuum dryer. Then 150 μL tetrahydrofuran was added to each of the dried tissue extracts, vortex-mixed for 10 min and evaporated to complete dryness in a nitrogen evaporator. MBDSTFA (100 μL) was added to each sample and derived at 60℃ for 30 min. The samples were vortexed for 30 s after derivation, for GC and MS analysis.

GC and MS analysis

Each of the samples was injected into an Agilent 6980 GC system equipped with a HP5MS capillary column (30 mm × 0.25 mm, i.d., 0.25 μm), and a quadrupole mass spectrometric detector (Agilent Technologies, Palo Alto, CA, USA). Helium was used as the carrier gas with a constant flow rate of 1.0 mL/min. One μL sample was injected into the Agilent 6980 GC system at 280℃. The column temperature was initially kept at 100℃ for 3 min, and then elevated to 220℃ at an increasing rate of 10℃ per min, followed by 10℃ per min to 280℃ for 5 min. Both of the interface and ion source temperature were 200℃. MS was conducted in an electron impact ionization mode at 70eV. Mass data were obtained in a full scan mode from m/z 100 to 600. Total ion chromatograms (TIC) and fragmentation patterns of GC were acquired using the GC/MSD ChemStation Software (Agilent Technologies, Palo Alto, CA, USA). Compounds were identified by comparing the mass spectrum with a standard mass spectrum in the National Institute of Standards and Technology (NIST) Mass Spectra Library. Peaks with a similarity index more than 70% were the assigned compound names, while those with a similarity index less than 70% were considered unknown metabolites[16]. Chromatograms were subjected to noise reduction prior to peak area integration. Any known artificial peaks due to derivatization of column bleed and BSTFA should be excluded from the data set. The resulting three-dimensional matrix included sample information, peak retention time, and peak area.

Statistical analysis

Data, normalized by dividing the sum of all peak areas in the sample (1 mg) before multivariate analysis, were expressed as mean ± SD and introduced into SPSS16.0 for Windows. Metabolite levels were compared by independent *t* test for the detection of significant differences in metastasis and non-metastasis groups. $P < 0.05$ was considered statistically significant. Principal components analysis (PCA) was performed to differentiate the metabolic patterns in metastasis and non metastasis groups. The differentiation performance was validated by the area under the curve (AUC) of receiver operating characteristic (ROC) curves. Similarly, metabolomic differences in metastasis group, non-metastasis group and control group were analyzed by *t* test and PCA.

RESULTS

The mean weight of mice in three groups was 23.81 ± 0.16, 23.87 ± 0.19 and 23.98 ± 0.19 g, respectively. Microscopy showed localized poorly-differentiated adenocarcinoma in all mice of non-metastasis and metastasis groups at the implanted sites. The average tumor weight was 4.28 ± 0.20 g in non-metastasis group and 4.30 ± 0.3 g in metastasis group, indicating that the tumor grows at a similar rate in the subcutis or stomach. However, the metastatic rate was significantly different when the tumor was implanted into the ectopic or orthotopic sites. Two thirds of the mice bearing an orthotopic tumor developed metastatic tumors in the region of lymph nodes, half in liver and one fourth in other organs. In contrast, no tumor metastasis was observed in the non-metastasis group after ectopic implantation. Macroscopic and histological examination showed no gastric cancer in the mice without tumor implantation. These results indicate that GC and MS are of a high reproducibility in the retention time of metabolites. Of the 152 signals detected in the tissue samples, some were not consistently found in other samples or could not be assigned to the unique metabolites because the abundance was too low. Fifty-eight signals could be identified by comparing a standard mass chromatogram with that in the NIST Library (Table 1). According to the NIST Mass Spectra Database, most of the chromatograms were identified as endogenous metabolites, such as amino acids, organic acids, inorganic acids, fatty acids, and pyrimidines, which were involved in several metabolic pathways, including glycolysis (lactic acid, alaline), serine metabolism (serine, phosphoserine), proline metabolism (proline), glutamic acid metabolism, glutamine metabolism, tricarboxylic acid (TCA) cycle (succinate), nucleotide metabolism (pyrimidine), fatty acid

Table 1 Tissue metabolites in mice of control, non-metastasis and metastasis groups

Peak No.	Percentage	RT (min) metabolites	Match
1	6.1128	Propanoic acid	87
2	7.9894	Acetic acid	97
3	9.8780	Silanol	93
4	10.9850	Lactic acid	88
5	11.4610	Propanedioic acid	96
6	12.1110	2-methylglutaconic acid	80
7	14.9138	Butanoic acid	90
8	15.4200	L-Alanine	89
9	15.9640	Glycine	92
10	16.4966	Urea	93
11	16.7655	Acetamide	81
12	17.5162	1, 2-Butanediol	93
13	17.9103	L-methioninamide	82
14	18.1660	L-Valine	80
15	18.3168	Ethanol	73
16	18.6734	5-Nonanol	74
17	19.1113	D, L-leucine	98
18	19.3427	Pyrimidine	93
19	19.4178	Dimethylglycine	96
20	19.7869	L-isoleucine	86
21	20.4186	Succinate	85
22	21.0066	Propanamide	87
23	21.5133	Butanedioic acid	81
24	21.8323	2-butenoic acid	95
25	23.0521	Hexanethioic acid	89
26	23.8090	Malic acid	92
27	24.3032	L-Proline	91
28	24.4971	Pyrrolidine	72
29	24.5909	L-Methionine	83
30	25.0350	L-Serine	97
31	25.5230	L-Threonine	84
32	25.9421	Silanol	95
33	26.2298	1, 3-propanediamine	91
34	26.4800	L-phenylalanine	96
35	26.5676	Tetrame-thylenediamine	83
36	27.1681	Hexanoic acid	97
37	27.3933	L-Aspartic acid	90
38	27.9563	Glutamine	94
39	28.1940	Phosphoserine	98
40	28.3942	Mannofuranose	83
41	28.7319	L-Glutamic acid	84
42	28.8383	Hypoxanthine	91
43	29.5264	Glucofuranose	75
44	29.7953	L-Lysine	95
45	30.0330	2-furancarboxylic acid	99
46	30.7462	Glucose	97
47	31.6532	L-histidine	87
48	31.7845	L-arginine	89
49	32.0472	L-Tyrosine	92
50	32.1598	D-ribofuranose	83
51	32.4726	Decanoic acid	81
52	32.6540	Hexanedioic acid	95
53	33.5861	5-pyrimidinecarboxylic acid	89
54	34.6932	Myo-inositol	94
55	34.9560	Hexadecanoic acid	90
56	35.0435	Docosanoic acid	92
57	35.3250	Octadecanoic acid	79
58	35.4439	Maltose	92

metabolism (docosanoic acid, and octadecanoic acid), and methylation(glycine).

The GC and MS data about tissue metabolites in metastasis and non-metastasis groups were analyzed by

Table 2 Different metabolites identified in metastasis and non-metastasis groups (mean ± SD)

No.	Metabolites	Metastasis group (n = 8)	Non-metastasis group (n = 8)	P	Fold
1	Lactate	4.194 ± 0.595	2.662 ± 0.218	0.000	1.58
2	Propanedioic	0.058 ± 0.004	0.0324 ± 0.002	0.000	1.80
3	Alanine	2.643 ± 0.145	1.903 ± 0.195	0.000	1.39
4	Glycine	0.762 ± 0.054	0.456 ± 0.172	0.001	1.67
5	Valine	1.036 ± 0.113	0.650 ± 0.047	0.000	1.59
6	Leucine	2.447 ± 0.306	1.756 ± 0.204	0.000	1.39
7	Pyrimidine	0.214 ± 0.030	0.166 ± 0.031	0.008	1.29
8	Dimethylglycine	0.372 ± 0.047	0.263 ± 0.020	0.000	1.42
9	Isoleucine	0.772 ± 0.087	0.992 ± 0.104	0.000	-1.29
10	Succinate	0.0794 ± 0.006	0.123 ± 0.018	0.000	-1.56
11	Propanamide	0.101 ± 0.007	0.147 ± 0.006	0.000	-1.45
12	Butanedioic	0.050 ± 0.008	0.073 ± 0.015	0.004	-1.46
13	Malicacid	3.053 ± 0.348	1.788 ± 0.116	0.000	1.71
14	Proline	1.173 ± 0.093	0.479 ± 0.072	0.000	2.45
15	Pyrrolidine	0.105 ± 0.016	0.067 ± 0.0083	0.000	1.57
16	Methionine	1.169 ± 0.099	1.518 ± 0.101	0.000	-1.30
17	Serine	1.742 ± 0.108	1.175 ± 0.074	0.000	1.48
18	Threonine	0.538 ± 0.040	0.632 ± 0.063	0.003	-1.17
19	Aspartic	5.870 ± 0.485	3.839 ± 0.453	0.000	1.53
20	Glutamine	0.090 ± 0.010	0.153 ± 0.016	0.000	-1.71
21	Phosphoserine	0.169 ± 0.0096	0.109 ± 0.012	0.000	1.56
22	Glutamate	3.533 ± 0.310	2.727 ± 0.200	0.000	1.30
23	Hypoxanthine	1.673 ± 0.150	1.841 ± 0.138	0.035	-1.10
24	Lysine	2.304 ± 0.137	1.808 ± 0.114	0.000	1.27
25	Glucose	1.338 ± 0.118	1.956 ± 0.142	0.000	-1.46
26	Arginine	1.342 ± 0.178	0.987 ± 0.088	0.000	1.36
27	Inositol	0.409 ± 0.030	0.314 ± 0.022	0.000	1.30
28	Docosanoic	0.123 ± 0.012	0.076 ± 0.016	0.000	1.61
29	Octadecanoic	0.756 ± 0.103	0.555 ± 0.134	0.005	1.36

P-value was calculated by student t test. $P < 0.05$ was considered statistically significant. Fold change with a positive value indicates a relatively higher level of metabolites while a negative value indicates a relatively lower level in metastasis group than in non-metastasis group.

Student's *t* test. Marker metabolites selected by Student's *t* test are presented in Table 2. Among these metabolites, proline was the most up-regulated tissue metabolite in metastasis group, which was 2.45-fold higher than that in non-metastasis group. Glutamine was the most down-regulated tissue metabolite in the metastasis group, which was 1.71-fold lower than that in the non-metastasis group. The lactic acid, L-alanine, L-valine, leucine, malic acid, L-aspartic acid, serine, phosphoserine, dimethylglycine, glycine, L-glutamic acid, L-lysine, myo-inositol, propanedioic acid, docosanoic acid, octadecanoic acid, arginine, pyrrodine, and pyrimidine were significantly up-regulated, while the glucose, succinate, L-isoleucine, L-methionine, propanamide, L-threonic acid, and butanedioic acid were remarkably down-regulated in the metastasis group compared to the non-metastasis group. The main metabolic pathways associated with metastasis of gastric cancer included glycolysis (lactic acid, alaline), serine metabolism (serine, phosphoserine), proline metabolism (proline), tricarboxylic acid (TCA) cycle (succinate, malic acid), fatty acid metabolism (docosanoic acid, and octadecanoic acid), and methylation(glycine).

The levels of lactic acid, propanedioic acid, L-alanine,

L-valine, glycine, leucine, malic acid, pyrrodine, serine, L-proline, L-methionine, L-threonine, L-aspartic acid, phosphoserine, L-glutamic acid, L-lysine, arginine, myo-inositol, docosanoic acid, and octadecanoic acid were higher in non-metastasis group and metastasis group than in control group, while the levels of glucose, L-isoleucine, succinate, and glutamine were lower in control group than in non-metastasis group and metastasis group.

PCA was used to identify the metabolic patterns associated with gastric cancer metastasis. The PCA scores of non-metastasis group and metastasis group were scattered into two different regions. ROC analysis, which was performed using the values determined by the first two components of the PCA model, confirmed the robustness of the PCA model. The sensitivity and specificity trade-offs were summarized for each variable with the AUC. The AUC value for the PCA model was 1.00, demonstrating a good differential value for metastasis of gastric cancer.

The PCA model was also validated by ROC analysis (AUC = 1.00), showing that the growth of human gastric cancer in mice was of distinct metabolic properties compared to that in the host.

DISCUSSION

Metastasis is one of the leading causes for poor prognosis of patients with gastric cancer[20]. The approaches used in tumor staging and prognosis assessment have a large number of limitations[21,22]. Recent advances in the field of metabolomics have provided the new opportunities to identify novel diagnostic markers and therapeutic targets for gastric cancer[6,11]. It is very important to establish a relevant model of cancer metastasis. Cell lines are often used as experimental models to investigate the genetic and biochemical alterations in tumor cells[23]. However, these models have limitations due to the lack of interactions between tumor cells and host[24]. Our previous study showed that human gastric cancer growing at orthotopic sites produces metastasis, and does not result in metastasis in SCID mice when it is implanted into the ectopic sites[19]. It has been shown that a mouse gastric cancer model develops metastasis similar to human tumor[19], which is in favor of research on gastric cancer metastasis. Consistent with our results, an orthotopically implanted pancreatic cancer model is a prerequisite for identifying a differential gene expression pattern unique to metastatic human pancreatic cancer[23].

In the present study, a highly metastasis model was successfully induced by orthotopic implantation of histologically intact tissue of SGC-7901 human gastric carcinoma while a non-metastasis model was established by subcutaneous implantation of the same cancer tissue in SCID mice. Tissue samples from gastric and subcutaneous tumors were derived from the same human gastric cancer with the same genetic background. The main difference in the two tumor models was their metastatic abilities. Therefore, metabolic analysis of these samples is valuable for the elucidation of metastatic mechanisms. To our knowledge, this is the first metabolomic investigation of gastric cancer metastasis in animal models. The comparative metabolomic strategy was used to identify the marker metabolites which were differentially expressed in animal models of human gastric cancer with distinct metastatic phenotypes. Of the 29 differential metabolites detected in the tumor models, 20 were up-regulated and 9 were down-regulated in the metastasis group compared to those in the non-metastasis group, indicating that these metabolites are involved in several metabolic pathways.

The lactic acid level was higher while the glucose level was lower in metastasis group than in non-metastasis group. Lactic acid is the end product of glycolysis. Increased glucose uptake and consumption are frequently observed in many cancer cells even under normoxic conditions, known as the Warburg effect. It has been demonstrated that the lactic acid level is increased in various metastatic cancers, including renal, uterine cervix, head and neck, colorectal cancers[25-27]. Moreover, the high lactate level in tumor tissue is associated with its metastasis and poor prognosis[27]. In fact, increased lactic acid produced by tumor cells can result in acid-mediated matrix degradation, T cell inactivation, up-regulation of VEGF and HIF-1 alpha, and enhancement of cell motility, thus providing favorable conditions for metastatic spread[25,27]. Therefore, high lactic acid levels reflect an increased energy demand for tumor progression.

In the present study, GC and MS showed that proline was the most up-regulated tissue metabolite, indicating that increased proline in metastatic gastric cancer tissue may be correlated with the increased turnover of extracellular matrix in metastatic cancer cells. Tumor cells need increased degradation of collagen during the process of invasion and metastasis, thus producing a large amount of proline. Pyrroline-5-carboxylic (P5C) is the precursor of proline and also its degradation product. Proline oxidase, also known as proline dehydrogenase, catalyzes the first step of proline to P5C in mitochondria and the latter is converted to proline by the cytosolic P5C reductase. It has been demonstrated that proline oxidase can be induced by p53 due to genotoxic stress and initiates apoptosis by the mitochondrial and death receptor pathways[28]. Proline is a stress substrate and matrix metalloproteinases can degrade collagen in the extracellular matrix. So far little attention has been paid to the correlation between proline metabolism and tumor progression. It has been shown that proline consumption is increased in patients with metastatic renal cancer[29]. Increased proline biosynthesis has been recently observed in metastatic breast cancer cell lines[30]. Furthermore, proline metabolism is linked with arginine and glutamate metabolism, TCA cycle and pentose phosphate pathway (PPP) due to P5C[28], suggesting that the significantly up-regulated metabolism of proline is highly correlated with cancer metastasis.

In this study, serine metabolism was involved in the metastatic process of gastric cancer, showing alterations in the pathway. The serine and phosphoserine increased 1.48-fold and 1.56-fold, respectively in metastatic tumors. It was reported that all the three genes involving the serine biosynthesis pathway, phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1),

and phosphoserine phosphatase (PSPH) are up-regulated in highly metastatic breast cancer cells[31], which is agreement with our results. Although most amino acids are increased in gastric cancer and colorectal cancer tissue compared with their adjacent normal tissue[17], little is known about the alterations related to metastatic behaviors. In the present study, the levels of leucine, valine glutamate and lysine were higher while the levels of methionine and threonine were lower in metastatic specimens, indicating that the demand for energy is increased in metastatic progression. Glycine can derive from serine synthesized by the glycolytic intermediate phosphoglycerate and its elevated levels may be associated with glycolysis. Sarcosine, a methylation derivative of the amino acid, glycine, is related to prostate cancer progression[6]. Dimethylglycine is another methylation product of glycine and how it is up-regulated remains unclear.

The disturbed TCA cycle, observed in a large number of tumors including gastric cancer, is considered to be related to carcinogenesis[17]. In this study, the TCA cycle intermediates such as succinate and malic acid were remarkably perturbed in metastatic specimens, suggesting that enhanced glycolysis contributes to metastatic progression.

In this study, the expression patterns of metastatic and non-metastatic human carcinoma were compared by metabolomic analysis, and several important metabolic pathways associated with metastasis of gastric cancer were identified. Of note, proline and serine metabolism were highlighted in this study. Further functional and clinical sample analysis of the metabolic pathways is needed to demonstrate their role in gastric cancer metastasis. The metabolic pathways may be exploited as biomarkers for gastric cancer progression.

COMMENTS

Background

Gastric cancer is the second cause of cancer-related death worldwide, and its metastasis is one of the leading causes of cancer-related death. The molecular mechanisms underlying gastric cancer metastasis are still not fully understood. Recent metabolomic studies have shown that metabolic alterations play a role in the biology of gastric cancer. The metabolic profiling of tumor tissue is used to elucidate the underlying mechanisms and identify the metabolomic markers of gastric cancer metastasis for improving its diagnostic and therapeutic strategies.

Research frontiers

Metabolomics, an OMIC science in systems biology, is the comprehensive and simultaneous profiling of metabolic changes occurring in living systems in response to genetic, environmental, or lifestyle factors. Recently, metabolomic method has shown great potentials in identifying the new diagnostic markers and therapeutic targets for cancers. However, metabolomic study on gastric cancer metastasis remains scarce.

Innovations and breakthroughs

Recently, most investigations have been focused on identifying the altered genes and proteins that play a role in cancer progression. In this study, the expression patterns of metastatic and non-metastatic human carcinoma were compared by metabolomic analysis, and several important metabolic pathways associated with metastasis of gastric cancer were identified. This is the first report on metabolomic investigation of gastric cancer metastasis.

Applications

The results of this study indicate that the metabolic pathways can be exploited as biomarkers for gastric cancer progression, which can be used in diagnosis and treatment of gastric cancer.

Terminology

Metabolomics, an OMIC science in systems biology, is the comprehensive and simultaneous profiling of metabolic changes occurring in living systems in response to genetic, environmental and lifestyle factors. Gas chromatography (GC) and mass spectrometry (MS) have been widely applied in metabolomic investigation because of their high sensitivity, peak resolution and reproducibility.

Peer review

To elucidate the underlying mechanisms of metastasis and identify metabolomic markers of gastric cancer metastasis, the authors performed GC/MS to identify the metabolomic difference in metastatic and non-metastatic lesions. The results indicate that proline and serine metabolism play an important role in gastric cancer metastasis, and that metabolic profiling of tumor tissue can provide new biomarkers for the treatment of gastric cancer metastasis.

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BRIEF ARTICLE

Impact of *KRAS* mutation and PTEN expression on cetuximab-treated colorectal cancer

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Abstract

AIM: To investigate the prognostic value of *KRAS* mutation, and phosphatase and tensin (PTEN) expression in Chinese metastatic colorectal cancer metastatic colorectal cancer (mCRC) patients treated with cetuximab.

METHODS: Ninety Chinese mCRC patients treated with cetuximab were evaluated for *KRAS* mutation and PTEN protein expression by DNA sequencing of codons 12 and 13 and immunohistochemistry, respectively. We then selected 61 patients treated with cetuximab, either in combination with chemotherapy, or alone as a second-line or third-line regimen to assess whether *KRAS* mutation or PTEN protein expression is associated with the response and the survival time of mCRC patients treated with cetuximab.

RESULTS: *KRAS* mutation was found in 30 (33.3%) tumor samples from the 90 patients, and positive PTEN expression was detected in 58 (64.4%) of the 90 patients. Among the 61 patients who were treated with cetuximab as a second-line or third-line regimen, the resistance to cetuximab was found in 22 patients with *KRAS* mutation and in 39 patients without *KRAS* mutation, with a response rate of 4.5% and 46.1% respectively ($P = 0.001$), a shorter median progression-free survival (PFS) time of 14 ± 1.3 wk and 32 ± 2.5 wk respectively ($P < 0.001$), a median overall survival (OS) time of 11 ± 1.2 mo and 19 ± 1.8 mo respectively ($P < 0.001$), as well as in 24 patients with negative PTEN expression and in 37 patients with positive PTEN expression respectively ($P < 0.001$), with a responsive rate of 4.2% and 48.6% respectively, a shorter median PFS survival time of 17 ± 2.0 wk and 28 ± 1.9 wk respectively ($P = 0.07$), and a median OS time of 11 ± 1.3 mo and 18 ± 1.9 mo respectively ($P = 0.004$). Combined KRAS mutation and PTEN expression analysis showed that the PFS and OS time of patients with two favorable prognostic factors were longer than those of patients with one favorable prognostic factor or no favorable prognostic factor ($P < 0.001$).

CONCLUSION: *KRAS* mutation and PTEN protein expression are significantly correlated with the response rate and survival time of Chinese mCRC patients treated with cetuximab.

Key words: Cetuximab; Metastatic colorectal cancer; *KRAS* mutation; Phosphatase and tensin protein expression

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INTRODUCTION

The incidence of colorectal cancer (CRC) has been increasing in the past decades and CRC is the third-leading cause of cancer-related deaths in China. During the past few years, several new biological agents have been evaluated in metastatic colorectal cancer (mCRC) with a remarkable anti-mCRC activity. Epidermal growth factor receptor (EGFR), one of the most promising targets, can activate the proliferation and prolong the survival time of cancer cells through the Ras/Raf/mitogen-activated protein kinase (MEK)/EPH receptor B2 (ERK) pathway or the phosphoinositide-3-kinase (PI3K)/PTEN/AKT pathway[1].

Cetuximab (Erbitux®, Merck KgaA, Darmstadt, Germany), a chimeric mouse/human antibody against the extracellular domain of EGFR, has a single-agent activity in mCRC refractory to irinotecan, oxaliplatin and fluoropyrimidines, and restores chemosensitivity in irinotecan-refractory mCRC patients[2-4]. However, only a small number of patients can benefit from cetuximab. The response rate to the combined cetuximab and irinotecan is about 23%[2]. Immunohistochemical studies showed that EGFR protein expression in CRC patients is not a useful predictor for the response to cetuximab[5,6].

Recent reports are available on the EGFR pathways, such as *KRAS*/BRAF/MAPKs, and on their potential correlation with cetuximab activity. *KRAS* somatic mutation occurs in approximately 40% of CRC patients. The negative predictive value of *KRAS* mutation has been confirmed in CRYSTAL study of first-line fluorouracil, leucovorin, and irinotecan (FOLFIRI) with or without cetuximab, demonstrating that only the patients with *KRAS* wild-type mutations benefit from cetuximab treatment[7-9].

Increasing interest in anti-EGFR therapy has been focused on another EGFR pathway, and PI3K/AKT/PTEN. PTEN encodes phosphatase with phosphatidylinositol-3, 4, 5-triphosphate (PIP-3) produced by the activity of PI3K as its major substrate. Loss of PTEN function increases PIP-3 concentration, and subsequent AKT hyperphosphorylation stimulates the proliferation of cancer cells[10].

It was reported that PTEN protein expression and *KRAS* mutation can predict the outcome of mCRC patients treated with cetuximab plus irinotecan, and negative PTEN expression in mCRC patients can predict the resistance to cetuximab plus irinotecan. Combined PTEN expression and *KRAS* mutational analysis can help to identify a subgroup of mCRC patients who have a greater chance of benefiting from EGFR inhibition[11].

KRAS and PTEN are the important molecular determinants of the EGFR downstream signal pathway and play an important role in anti-EGFR therapy in Western countries. However, little is known about the correlation between *KRAS* mutation and PTEN protein expression with the activity of anti-EGFR mono-antibody in Asian populations. This retrospective study was to evaluate the prognostic value of EGFR downstream cascade members, *KRAS* and PTEN, in Chinese mCRC patients treated with cetuximab plus chemotherapy.

MATERIALS AND METHODS

Patients

We retrospectively assessed 90 mCRC patients (59 males and 31 females with a median age of 53.0 ± 13.9 years) treated with cetuximab in Sun Yat-Sen University Cancer Center and Beijing Cancer Hospital from June 2000 to August 2008. The patients had histologically proven colorectal adenocarcinoma and the tumor response to cetuximab treatment was evaluable. Tissue samples of primary colorectal tumor were taken. *KRAS* mutation and PTEN protein expression in the patients were analyzed. Of the 90 patients, 3 received cetuximab monotherapy, 58 received cetuximab in combination with irinotecan-based chemotherapy, and 29 received cetuximab in combination with oxaliplatin-based chemotherapy. Cetuximab was administered as the first- fourth lines of treatment in 29, 23, 28, and 10 patients, respectively (Table 1). Paraffin-embedded tumor tissue samples from 100 mCRC patients (69 males and 31 females with a mean age of 50.5 ± 12.1 years) not treated with cetuximab were used for gene analysis. Furthermore, *KRAS* mutation in these patients was analyzed to confirm the mutation frequency of *KRAS*.

Skin toxic effects were assessed according to the National Cancer Institute Common Toxicity Criteria, version 2. Tumor response to cetuximab was evaluated by computerized tomodensitometry according to the response evaluation criteria in solid tumors (RECIST) and classified as complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). Patients with CR or PR and SD or PD were classified as responders and non-responders, respectively, for the analyses.

Nucleotide sequence analysis

KRAS mutation was analyzed by extracting genomic DNA from the paraffin-embedded tissue sections with a QIAamp DNA mini kit (Qiagen, Berlin, Germany) according to its manufacturer's instructions. Exon 1 of the *KRAS* gene (GenBank, L00045, nt 102 to 235) was then directly PCR-amplified in a thermal cycler. The sequences of primers used for *KRAS* analysis (codons 12 and 13) are identical to those used in a previous study[12]. PCR condi-

Table 1 Data about patients enrolled in this study

Characteristics	Patients, *n* (%)
Sex	
Male	59 (65.6)
Female	31 (34.4)
Age (yr)	
Median	53
Range	23-75
Tumor site	
Colon	39 (43.3)
Rectal	51 (56.7)
Combined chemotherapy	
Irinotecan-based	58 (64.4)
Oxaliplatin-based	29 (32.2)
Monotherapy	3 (3.3)
Cetuximab use	
First line	29 (32.2)
Second line	23 (25.6)
Third line	28 (31.1)
Fourth line or more	10 (11.1)
Response status	
Complete response	3 (3.3)
Partial response	31 (34.4)
Stable disease	35 (38.9)
Progressive disease	21 (23.3)

tions were as follows: 1 cycle at 95℃ for 9 min, 45 cycles at 94℃ for 1 min, at 55℃ for 1 min, and at 72℃ for 1 min, followed by 1 cycle at 72℃ for 5 min. After confirmed by agarose electrophoresis and ethidium bromide staining, the PCR products were purified and automatically sequenced with an ABI PRISM 3730 (Applied Biosystems, California, USA), then analyzed with Chromas software version 2.0 (Gene Codes Corporation, USA). All sequencing reactions were performed in both forward and reverse directions, by independent PCR.

PTEN protein expression

PTEN protein expression in 3-mm thick tissue sections was evaluated using the anti-PTEN clone 6H2.1 (Millipore Company, Massachusetts, USA). The sections were deparaffinized and hydrated by passing through xylene and a graded series of ethanol. Endogenous peroxidase activity of the sections was blocked by incubating them in 0.3% hydrogen peroxide for 20 min. Antigen was retrieved for 30 min at 98℃ in a 0.01 mol/L sodium citrate buffer (pH 6.4) in a microwave oven. After blocked for 30 min in 0.75% normal goat serum, the sections were incubated with 6H2.1 at a dilution of 1:100 overnight at 4℃, washed in PBS, and then incubated with biotinylated goat anti-mouse IgG followed by avidin peroxidase using a Vectastain ABC elite kit (Vector Laboratories, California, USA). The reaction products were counterstained with hematoxylin, and the sections were evaluated under a light microscope. PTEN protein expression was detected mainly in cytoplasm, although nuclear signals were occasionally observed as previously reported[13]. The intensity of reaction was assessed as a score of either 1+, 2+ or 3+, and the percentage of positive cells was classified into three groups (0%-25%, 25%-50% and > 50% of cells) and assigned to 1, 2 or 3 points, respectively. Tumors producing more than 4 points were considered PTEN-positive tumors according to the two values for the products. The evaluation was performed without knowledge of the clinical data or the results of other analyses.

Statistical analysis

Fisher's exact test was used to calculate the *P* values for *KRAS* mutation, PTEN expression, skin toxicity, and response to cetuximab. PFS time was calculated as the period of time from the first day of cetuximab treatment to the date of tumor progression, the date of death due to any factor, or the date of last follow-up. OS time was calculated as the period of time from the first day of cetuximab treatment until death due to any factor, or until the date of last follow-up. Cox proportional hazards regression model was used in survival analysis. PFS curves for PTEN expression and *KRAS* mutation were plotted using the Kaplan-Meier method, and the difference in biomarkers was evaluated using the log-rank test. Analysis was carried out with the SPSS software version 16.0 (SPSS Company, USA). $P < 0.05$ was considered statistically significant.

RESULTS

The clinical characteristics of mCRC patients treated with cetuximab are summarized in Table 1. The median follow-up time was 13.5 mo. Of the 90 patients, 34 (37.8%) had a response to cetuximab plus chemotherapy. The median PFS and OS time was 22 wk (range 8-129 wk), and 11 mo (range 2-48 mo), respectively. *KRAS* mutation was found in 30 (33.3%) tumor tissue samples from the 90 patients. Of the 30 tumor tissue samples, 25 and 5 were the single amino acid substitutions in codons 12 and 13, respectively. The *KRAS* mutations on codon 12 predominantly involved the second base of the codon, with the presence of GaT mutation (GGT-GaT, Gly-Asp, G12D), GtT mutation (GGT-GtT, Gly-Val, G12D), aGT mutation (GGT-aGT, Gly-Ser, G12C), and tGT mutation (GGT-tGT, Gly-Cys, G12C), in 16 (53.3%), 6 (20.0%), 2 (6.7%), and 1 (3.3%) out of the 30 patients, respectively. The *KRAS* mutations on codon 13 corresponded to the transition G-a at the second base of the codon (GGC-GaC, Gly-Asp, G13D). Positive and negative PTEN expression was detected in 58 (64.4%) and 32 (36.6%) of the 90 patients, respectively (Figure 1).

Of the 100 mCRC patients used for confirmation of *KRAS* mutation, 29 (29%) displayed *KRAS* mutation on codons 12 and 13, which was not significantly different from that in the 90 patients ($P = 0.213$). The *KRAS* mutations from GGT to GaT (Gly-Asp) and GtT (Gly-Val) on codon 12 accounted for 58.6% (17 of 29) and 20.7% (6 of 29) of the specified mutations, respectively. Mutations from GGC to GaC (Gly-Asp) occurred in 20.7% (6 of 29) of *KRAS* mutations on codon 13. The total mutation rate was 31% in 190 patients evaluated for *KRAS* mutations.

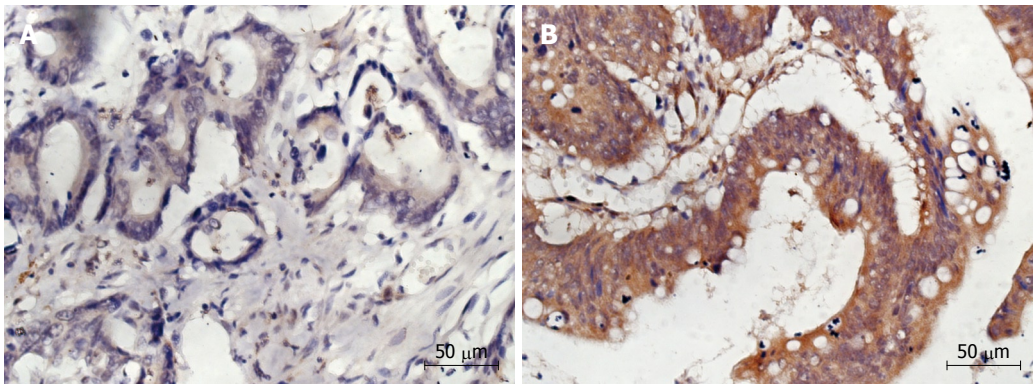

Figure 1 Immunohistochemistry showing phosphatase and tensin protein expression. A: Patients with absent phosphatase and tensin (PTEN) expression (HE stain, × 400); B: Patients showing positive PTEN expression (HE stain, × 400).

Table 2 Correlation of *KRAS* gene status and phosphatase and tensin protein expression with clinical response to cetuximab in previously treated colorectal cancer patients *n* (%)

	KRAS			PTEN		
	Mutation	No mutation	*P*	Positive	Negative	*P*
CR	0 (0)	2 (5.1)	0.001	2 (5.4)	0 (0)	0.001
PR	1 (4.5)	16 (41.0)		16 (43.2)	1 (4.2)	
SD	7 (31.8)	17 (43.6)		10 (27.0)	14 (58.3)	
PD	14 (63.6)	4 (10.3)		9 (24.3)	9 (37.5)	
Total	22 (100)	39 (100)		37 (100)	24 (100)	

CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; PTEN: Phosphatase and tensin.

We attempted to assess whether the *KRAS* mutation, PTEN protein expression or skin toxicity is associated with the clinical response of mCRC to cetuximab. Sixty-one patients treated with cetuximab plus chemotherapy as a second-, third-, or greater-line regimen were enrolled for analysis. The *KRAS* mutation was detected in 22 patients (36.1%). One of the 22 patients with *KRAS* mutation responded to cetuximab, and 18 of the 39 patients without *KRAS* mutation responded to cetuximab, with a response rate of 4.5% and 46.1%, respectively ($P = 0.001$). Positive PTEN expression was detected in 37 (60.7%) out of the 61 patients. Eighteen of the 37 patients with normal PTEN expression and one of the 24 patients with negative PTEN expression responded to cetuximab, with a response rate of 48.6% and 4.2%, respectively ($P = 0.001$). The absence of *KRAS* mutation and the presence of PTEN protein expression were significantly associated with a high response rate to cetuximab (Table 2). Meanwhile, combined *KRAS* mutation status and PTEN expression analysis showed that 24 (39.3%) of the 61 patients had no *KRAS* mutation and positive PTEN expression, with a remarkably higher effective rate than other patients (70.8% *vs* 5.4%, $P < 0.001$). Fisher's exact test also showed that the skin toxicity was significantly associated with a high response rate to cetuximab ($P < 0.001$).

Furthermore, we assessed whether *KRAS* mutation, PTEN protein expression, or skin toxicity is associated with the PFS and OS time in the 61 patients. Univariate analysis of PFS time showed that *KRAS* mutation was significantly associated with a short PFS time ($P < 0.001$). The median PFS time of mCRC patients without and with *KRAS* mutation was 32 ± 2.5 wk and 14 ± 1.3 wk, respectively. The PFS time was longer in mCRC patients with positive PTEN protein expression than in those with negative PTEN protein expression (28 ± 1.9 wk *vs* 17 ± 2.0 wk, $P = 0.07$) (Figure 2). No difference was found in the median PFS time between the patients with and without skin toxicity (27 ± 2.9 wk *vs* 18 ± 1.7 wk, $P = 0.113$). The median OS time of mCRC patients without *KRAS* mutation was significantly longer than that of those with *KRAS* mutation (19 ± 1.8 mo *vs* 11 ± 1.2 mo, $P < 0.001$). The median OS time of mCRC patients with positive PTEN expression was significantly longer than that of those with negative PTEN expression (18 ± 1.9 mo *vs* 11 ± 1.3 mo, $P = 0.004$) (Figure 3). The median OS time of mCRC patients with skin toxicity was longer than that of those without skin toxicity (17 ± 1.5 mo *vs* 11 ± 1.0 mo, $P = 0.025$). Multivariate analysis of the 61 patients showed that both *KRAS* mutation and PTEN protein expression were closely related with a shorter OS time ($P < 0.001$). No correlation was found between skin toxicity and *KRAS* mutation or PTEN protein expression.

In this study, the absence of *KRAS* mutation and positive PTEN expression were found to be two favorable prognostic factors for mCRC patients. Combined *KRAS* mutation and PTEN expression analysis showed that the median PFS time of mCRC patients with the two favorable prognostic factors was longer than that of those with only one favorable prognostic factor or with no favorable prognostic factor (32 ± 2.5 wk *vs* 17 ± 1.9 wk and 11 ± 1.8 wk, $P = 0.001$) (Figure 4). The median OS time of these three groups of mCRC patients was 22 ± 2.3 mo, 11 ± 1.5 mo, and 6 ± 1.0 mo, respectively ($P < 0.001$).

DISCUSSION

KRAS serves as a mediator for the extracellular ligand

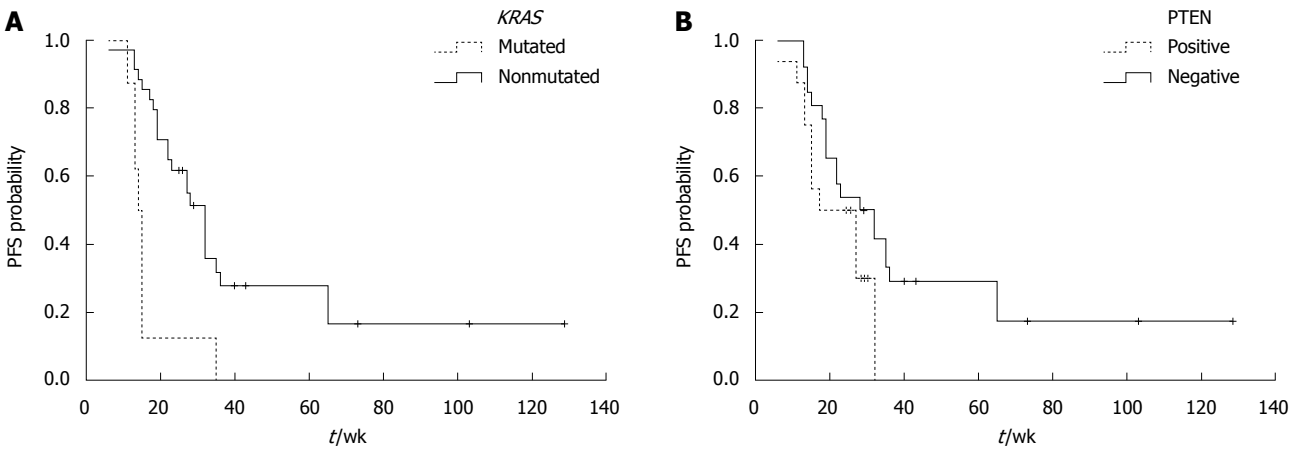

Figure 2 Progression-free survival time of patients with or without *KRAS* mutation (A) and phosphatase and tensin protein expression (B). A: $P < 0.001$; B: $P = 0.07$. PFS: Progression-free survival.

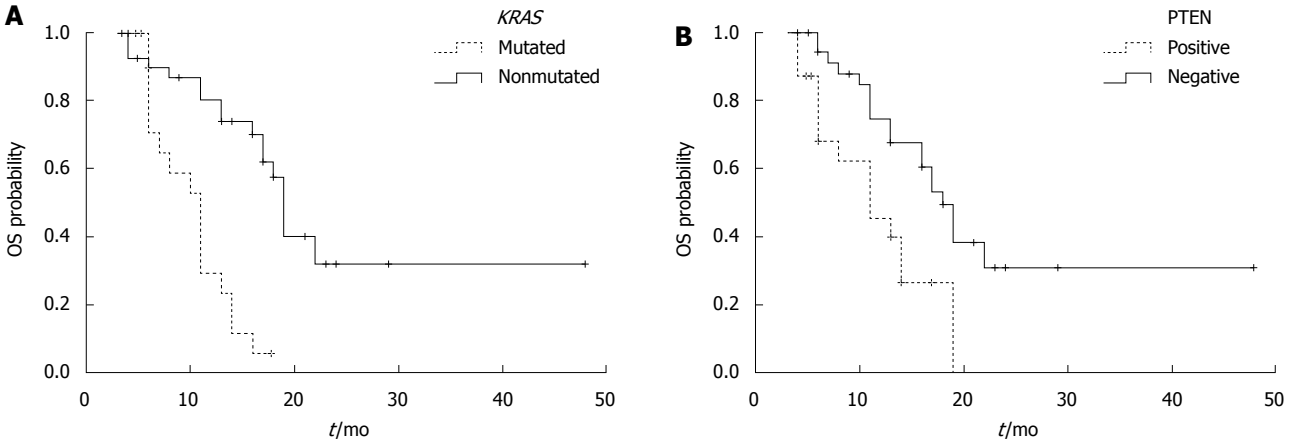

Figure 3 Overall survival time of patients with or without *KRAS* mutation (A) and phosphatase and tensin protein expression and skin toxicity (B). A: $P < 0.001$; B: $P = 0.004$. OS: Overall survival.

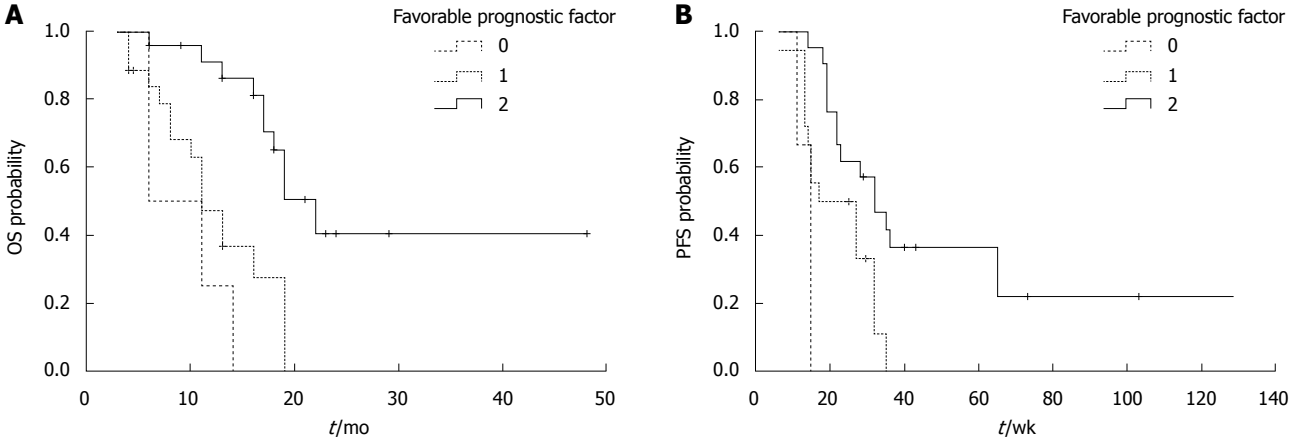

Figure 4 Overall survival and progression-free survival time of patients with or without favorable prognostic factors. A: $P < 0.001$; B: $P = 0.001$. 0: *KRAS* mutation and loss of phosphatase and tensin (PTEN); 1: Either no *KRAS* mutation or normal PTEN expression; 2: No *KRAS* mutation and normal PTEN expression. OS: Overall survival; PFS: Progression-free survival.

binding and intracellular transduction signals from EGFR to nuclei. RASCAL study evaluated the *KRAS* mutational in 2721 CRC patients from 22 centers and demonstrated that *KRAS* mutation is closely related with the progression and outcome of CRC[14]. In the present study, *KRAS* mutation was observed in about 40% (20%-50%) of spo-

radic CRC patients. Up to 90% of activating *KRAS* mutations were detected at codons 12 and 13, less than 70% of activating *KRAS* mutations were frequently detected at codon 12, and 70% of activating *KRAS* mutations were detected at codon 13. It has been shown that the most frequent types of *KRAS* mutation in CRC patients are GGT-GaT (Gly-Asp) and GGT-GtT (Gly-Val) transitions at codon 12[15]. In our study, 31% of *KRAS* mutations occurred in 190 CRC patients, with most specified *KRAS* mutations found at the second base of codon 12. The most common *KRAS* mutation at codon 12 was GGT to GaT. These findings are consistent with those in a previous study[15], indicating that the frequency or type of *KRAS* mutations is not different in Chinese and Western CRC patients.

It has been demonstrated that the benefit of cetuximab treatment in combination with first-line chemotherapy is restricted to CRC patients with *KRAS* wild-type mutations[9,16]. The relation between *KRAS* mutation and response to anti-EGFR therapy has also been intensively studied[11,13,17]. Lièvre *et al*[17] found that CRC patients with *KRAS* mutations are resistant to cetuximab therapy and have an unfavorable prognosis. It was reported that the PFS and OS time are shorter in CRC patients with *KRAS* mutations than in those with wild-type *KRAS* mutations[7,8]. In our study, *KRAS* mutation was found to be a powerful predictor for the resistance to cetuximab, the response rate of CRC patients with *KRAS* and *KRAS* wild-type mutations was 4.5% and 46.1%, respectively. The PFS and OS time of CRC patients with *KRAS* mutations was shorter than that of those without *KRAS* mutations. It has also been shown that treatment with tyrosine kinase inhibitors is not effective for non small cell lung cancer patients with *KRAS* mutations[18,19], indicating that *KRAS* mutations play a fundamental role in the EGFR pathway, thus rendering EGFR inhibitors ineffective[20].

In our study, the response rate of CRC patients with *KRAS* wild-type mutations was only 46.1%, indicating that there must be other unidentified genetic determinants of resistance to cetuximab therapy for CRC. The PI3K/PTEN/AKT pathway is on the other side of the two EGFR pathways. PTEN is a tumor suppressor protein that regulates the PI3K/AKT signal transduction. Its loss is associated with intrinsic activation of the AKT pathway and confers resistance to inhibitors of the HER family[21]. Thomas and Grandis demonstrated that PTEN is lost in 30% of sporadic CRC patients[22]. PTEN protein expression may be another molecular predictor for the response to cetuximab. Frattini *et al*[13] reported that loss of PTEN protein expression is associated with the lack of response to cetuximab. Sartore-Bianchi *et al*[10] showed that loss of PTEN protein expression is associated not only with the lack of objective tumor response, but also with a shorter OS time of mCRC patients treated with cetuximab. Loupakis *et al*[11] revealed that combined PTEN expression and *KRAS* mutation analysis helps identify a subgroup of mCRC patients who have a greater chance of benefiting from EGFR inhibition. In our study, positive PTEN expression was detected in 64.4% of mCRC patients, which is consistent with previous reports[10-13]. In this study, the response to cetuximab was significantly correlated with PTEN protein expression. The PFS and OS time of mCRC patients with negative PTEN protein expression was shorter than that of those with positive PTEN expression. The response rate of the 24 mCRC patients with no *KRAS* mutation and positive PTEN expression was substantially higher than that of those with *KRAS* mutation and positive PTEN expression. Combined *KRAS* mutation and PTEN protein expression analysis showed that the PFS and OS time of mCRC patients with two favorable prognostic factors was longer than that of those with one favorable prognostic factor or no favorable prognostic factor, indicating that a comprehensive analysis of *KRAS* mutation and PTEN protein expression is a better predictor for the clinical outcome of mCRC patients treated with cetuximab, which requires further confirmation in a prospective series.

It has been shown that skin toxicity is significantly associated with the response to cetuximab and OS time of mCRC patients[2,5,23,24], which is consistent with the findings in our study. In our study, the response rate of mCRC patients with skin toxicity was higher than that of those without skin toxicity, and the OS time of mCRC patients with skin toxicity was also longer than that of those without skin toxicity. However, univariate analysis showed that skin toxicity was only associated with OS time, while multivariate analysis showed that *KRAS* mutation and PTEN protein expression were the significant risk factors for OS time, indicating that skin toxicity alone is insufficient to predict the outcome of mCRC patients treated with cetuximab. Moreover, *KRAS* mutation and PTEN protein expression was detected before cetuximab treatment and can thus be included in the algorithm of treatment decision[17].

To our knowledge, this is the first study on *KRAS* mutation and PTEN protein expression in Chinese mCRC patients. Other markers were also identified in our study, which can be used to select mCRC patients who are likely to benefit from cetuximab treatment, showing that *KRAS* mutation and PTEN protein expression in Chinese mCRC patients are similar to those in other populations. In this study, skin toxicity was insufficient to predict the outcome of mCRC patients treated with cetuximab, and *KRAS* mutation and PTEN protein expression were significantly associated with the response rate to cetuximab and survival time of these patients.

In conclusion, combined *KRAS* mutation and PTEN protein expression analysis is a better predictor for the clinical outcome of mCRC patients treated with cetuximab. Prospective studies with a large number of patients are required to further confirm the results of our study.

COMMENTS

Background

The incidence of colorectal cancer (CRC) has been increasing in past decades

and CRC is presently the third-leading cause of cancer-related deaths in China. During the past few years, several new biological agents have been evaluated in metastatic colorectal cancer (mCRC) with remarkable clinical activity. Cetuximab is an important biological agent used in treatment of mCRC, but it is effective only in a subset of mCRC patients.

Research frontiers

Studies have shown that *KRAS* mutation and phosphatase and tensin (PTEN) protein expression are associated with the response to cetuximab and may have a prognostic value. However, the situation in Asian patients is unknown. The authors evaluated the prognostic value of *KRAS* mutation and PTEN protein expression in Chinese mCRC patients treated with cetuximab plus chemotherapy.

Innovations and breakthroughs

To the authors' knowledge, this is the first study on *KRAS* mutation and PTEN protein expression in Chinese mCRC patients. The results of this study show that *KRAS* mutation and PTEN protein expression in Chinese mCRC patients are significantly correlated with the response rate and survival time of patients treated with cetuximab. A comprehensive analysis of *KRAS* mutation and PTEN protein expression is a better predictor for the clinical outcome of patients treated with cetuximab.

Applications

KRAS mutation and PTEN protein expression can be used to select Chinese mCRC patients who are likely to benefit from cetuximab treatment.

Terminology

Epidermal growth factor receptor (EGFR), one of the most promising targets, can activate the proliferation and prolong the survival time of cancer cells through the Ras/Raf/mitogen-activated protein kinase (MEK)/EPH receptor B2 (ERK) or the phosphoinositide-3-kinase (PI3K)/ PTEN /AKT pathway. *KRAS* serves as a mediator for the extracellular ligand binding and intracellular signal transduction from EGFR to nuclei. PTEN is a tumor suppressor protein that regulates the PI3K/AKT signal transduction. Its loss is associated with the intrinsic activation of the AKT pathway.

Peer review

The manuscript describes the impact of *KRAS* mutation and PTEN protein expression, either alone or in combination, on cetuximab-treated Chinese colorectal cancer patients. The manuscript targets a topic that is of scientific and clinical interest.

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BRIEF ARTICLE

Irinotecan-involved regimens for advanced gastric cancer: A pooled-analysis of clinical trials

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Abstract

AIM: To assess the efficiency and toxicities of irinotecan (CPT-11)-involved regimens in patients with advanced gastric cancer.

METHODS: Randomized phases Ⅱ and Ⅲ clinical trials on chemotherapy for advanced gastric cancer were searched from MEDLINE, EMbase, Cochrane Controlled Trials Register, and EBSCO. Relevant abstracts were manually searched. A total of 657 patients were analyzed for their overall response rate (ORR), time to treatment failure (TTF), overall survival (OS) rate, and toxicities. Overall survival rate, reported as hazard ratio (HR) with 95% CI, was used as the primary outcome measure.

RESULTS: Four randomized controlled trials on chemotherapy for advanced gastric cancer were detected. The CPT-11-containing combination chemotherapy was not significantly advantageous over the non CPT-11-containing combination chemotherapy for OS rate (HR = 1.12, 95% CI: 0.92-1.36, P = 0.266) and ORR [risk ratio (RR) = 1.23, 95% CI: 0.71-2.14, P = 0.458]. However, the CPT-11-containing combination chemotherapy was significantly advantageous over the non CPT-11-containing combination chemotherapy for TTF (HR = 1.35, 95% CI: 1.12-1.64, P = 0.002). Grade 3/4 haematological toxicity (thrombocytopenia: RR = 0.20, 95% CI: 0.09-0.48; P < 0.001) and gastrointestinal toxicity (diarrhea: RR = 4.09, 95% CI: 2.42-6.93, P < 0.001) were lower in patients with advanced gastric cancer after CPT-11-containing combination chemotherapy than after non CPT-11 -containing combination chemotherapy.

CONCLUSION: CPT-11-containing combination chemotherapy is advantageous over non CPT-11 -containing combination chemotherapy for TTF with no significant toxicity. CPT-11-containing combination chemotherapy can be used in treatment of advanced gastric cancer.

Key words: Meta-analysis; Advanced gastric cancer; Chemotherapy

Peer reviewer: Susumu Ohwada, Associate Professor, Department of Surgery, Gunma University Graduate School of Medicine, 3-39-15 Shoma-Machi, Maebashi 371-8511, Japan

Wang DL, Gu DY, Huang HY, Xu Z, Chen JF. Irinoteca-involved regimens for advanced gastric cancer: A pooled-analysis of clinical trials. *World J Gastroenterol* 2010; 16(46): 5889-5894 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i46/5889.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i46.5889

INTRODUCTION

Although the incidence of gastric cancer has been sharply declined during the second half of the 20th century, it remains the second leading cause of cancer-related death in the world[1]. The morbidity and mortality rate of gastric

cancer increase with age. The most effective treatment for gastric cancer is radical gastrectomy. A substantial number of patients, however, eventually die of recurrence after curative resection. Although systemic chemotherapy can improve the quality of life in patients with gastric cancer[2], the outcome of patients with unresectable gastric cancer is still extremely poor with a median survival time of 3-5 mo after the best supportive care[2-4].

Randomized clinical trial data demonstrate that the survival rate and quality of life are better in patients with advanced gastric cancer after chemotherapy than in those after the best supportive care[5]. Over the years, a number of single-agent chemotherapy trials have confirmed that gastric cancer is a relatively "chemosensitive" disease[6-9]. It is, therefore, necessary to investigate different combination chemotherapies, both in phase Ⅱ and randomized phase Ⅲ trial settings.

First line chemotherapy usually consists of different combination regimens with 5-fluorouracil (5-FU) and cisplatin, including FP (5-FU and cisplatin) and ECF (epirubicin, cisplatin, and 5-FU). It has been shown that the response rate and progression-free survival rate are better for patients with gastric cancer after FP therapy than after 5-FU or other combination therapies[10,11]. The additional survival advantage yielded by these combination therapies appears to be marginal. However, no standard regimen has yet been established. Thus, it is necessary to develop new agents and combination regimens to achieve greater survival benefits in advanced or recurrent unresectable gastric cancer. Since 2005, combination chemotherapy for advanced gastric cancer has been focused on the integration of other chemotherapy agents, including docetaxel, irinotecan, oxaliplatin, capecitabine, and S-1.

Irinotecan (CPT-11) is a water-soluble camptothecin derivative. CPT-11 and its active metabolite (SN-38) bind reversibly to the topoisomerase I-DNA complex and induce cancer cell death by preventing relegation of single-strand DNA breaks[12,13]. It has been shown that CPT-11 acts as a single agent in oesophago-gastric cancer. It was reported that the overall response rate of advanced gastric cancer patients to chemotherapy is 16%-20%[14,15]. It has been reported that CPT-11 in combination with leucovorin/5-FU (ILF)[16] or cisplatin (IP)[17] exhibits its antitumor activity against advanced gastric cancer.

Several phases Ⅱ and Ⅲ randomized trials are available on CPT-11-containing or non CPT-11-containing combination chemotherapy for advanced gastric cancer[18-21]. The meta-analysis in this study was to compare the two therapies by evaluating their clinical efficiency and toxicities.

MATERIALS AND METHODS

Literature search

Trials were searched from MEDLINE, OLD MEDLINE, CancerLit, EMbase, and ISI Web of Science, incorporating Science Citation Index, Technology Proceedings, and Current Contents Databases as far back as they go. References of selected articles and previous systematic reviews were also searched for any other relevant trials (Figure 1). The search strategy included the following key words variably combined: metastatic, metastasis, gastric, cancer, CPT-11, randomized, trial.

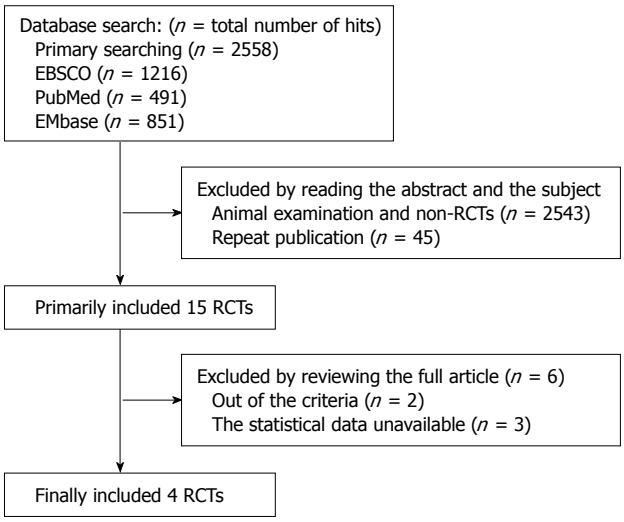

Figure 1 Article search and riddling progression. RCTs: Randomized controlled trials.

Two independent reviewers assessed the eligibility of the searched abstracts. If the eligibility of the abstract was unclear, the full article was retrieved for clarification. Any disagreements were solved by discussion. The selection criteria included study design (randomized or controlled trials), participants (patients with histologically confirmed advanced or recurrent adenocarcinoma, including diffuse type, intestinal type of the stomach or gastroesophageal junction). The exclusion criteria were nonrandomized trials, animal examination, single-arm phase Ⅱ trial, or adequate statistical analysis with information missed. Care was taken to include only primary data or data that superseded earlier works. The deadline for trial inclusion was November 6, 2009.

Statistical methods

Overall survival rate was used as the primary outcome measure. Secondary outcome measures evaluated were overall response rate (ORR: number of partial and complete responses) and toxicities (published by the authors with the most frequently reported events analyzed). Hazard ratio (HR) and 95% CI as relevant effect measures were estimated directly or indirectly from the given data. Appropriate data, such as log-rank test *P* value, were extracted for the estimation of the log HR and its variance as previously described[22,23]. Summary statistical data were extracted from the published trials according to the standard methods for survival end points, with HR and CI as preferred sources for estimation, and log-rank *P* value/event count as a second choice[22]. Standard techniques for meta-analysis[24] were used to calculate the pooled estimates. All analyses were conducted using the Stata software version 8.2 (Stata Corp LP, College Station, TX). All tests were two sided. Fixed-(primarily) and random-effect model methodology was applied. All reported *P* values

Table 1 Trials comparing irinotecan-containing and nonirinotecan-containing combination chemotherapies, treatment schedule and quality of each trial

Study	Regimen	Patients (*n*)	RR (%)	Survival (mo)	
				Progression free	Overall
Bouché *et al*[18]	FU	45	13.0	3.2	6.8
	CF	44	27.0	4.9	9.5
	IF	45	40.0	6.9	11.3
Moehler *et al*[19]	ILF	56	42.9	4.5	10.8
	ELF	58	24.1	2.3	8.3
Dank *et al*[20]	IF	170	31.8		9.0
	CF	163	25.8		8.7
Nakashima *et al*[21]	IP	44	47.0	5.7	14.8
	SP	32	80.0	7.8	15.6

FU: Fluorouracil; CF: Cisplatin and FU; IF: Irinotecan and FU; RR: Response rate; ILF: Irinotecan, leucovorin and FU; ELF: Etoposide, leucovorin and FU; IP: Irinotecan and cisplatin; SP: S-1 and cisplatin.

resulted from two-sided versions of the respective tests.

In consideration of possible heterogeneity across the studies, a statistical test for heterogeneity was performed as previously described[25]. $P < 0.05$ was considered statistically significant for the heterogeneity. A fixed effect approach was adopted unless there was evidence for significant unexplained heterogeneity, in which a random effect approach was used. In the absence of heterogeneity, the two methods provided identical results, because the fixed-effect model using the Mantel-Haenszel's method assumes that studies are sampled from populations with the same effect size, making an adjustment to the study weights according to the in-study variance, whereas the random-effect model using the DerSimonian and Laird's method assumes that studies are taken from populations with varying effect sizes, calculating the study weights both from in-study and between-study variances, considering the extent of variation or heterogeneity. Funnel plots and Egger's linear regression test were used to show the potential publication bias in diagnosis of advanced gastric cancer[26].

RESULTS

The results of 4 randomized phases Ⅱ and Ⅲ trials, including 2 Europe randomized phase Ⅱ trials[18,19], 1 Europe randomized phase Ⅲ trial[20], and 1 Japanese study[21], that have been published or presented at major international meetings, were included in this analysis. These studies included 657 patients with metastatic gastric cancer, of whom 315 (48%) received the CPT-11-containing combination chemotherapy. Treatment schedule and quality of each trial were evaluated (Table 1).

Overall survival time

The overall survival rate was reported in the 4 trials[18-21], during which 315 patients received CPT-11-containing combination chemotherapy and 342 patients received non CPT-11-containing combination chemotherapy. However, only 2 trials reported the HR. The other trials showed that the overall survival time of patients with gastric cancer was 11.3-14.8 mo after CPT-11-containing combination chemotherapy and 6.8-15.6 mo after non CPT-1-containing combination chemotherapy. No striking inter-study heterogeneity was found ($P = 0.535$, $I^2 = 0.0\%$) in the 4 trials. Meta-analysis of the pooled data demonstrated that the overall risk of death was not different between the two chemotherapies (HR = 1.12, 95% CI: 0.92 -1.36, $P = 0.266$, Figure 2).

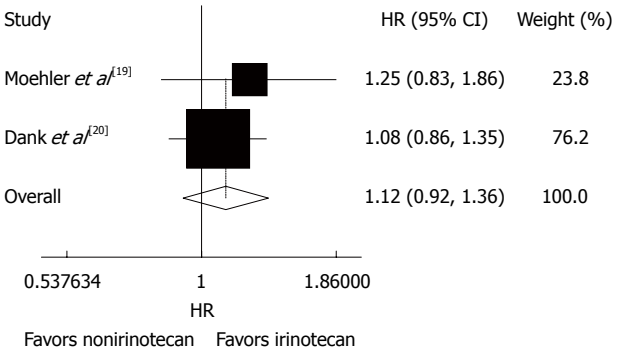

Figure 2 Overall survival rate of patients with advanced gastric cancer after irinotecan-containing and nonirinotecan-containing combination chemotherapies. HR: Hazard ratio.

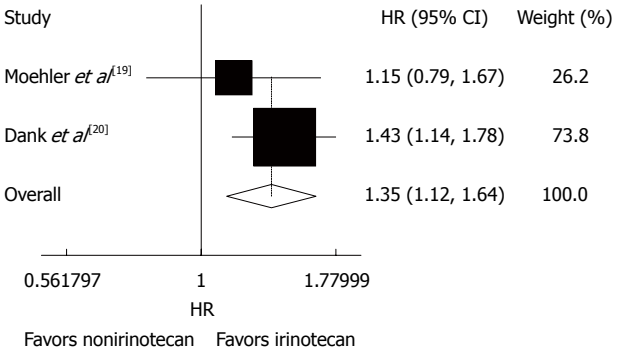

Figure 3 Time to treatment failure of patients with advanced gastric cancer irinotecan-containing and nonirinotecan-containing combination chemotherapies. HR: Hazard ratio.

Time to treatment failure

Two trials[19,20] analyzed the impact of time to treatment failure (TTF) with no striking inter-study heterogeneity ($P = 0.327$, $I^2 = 0.0\%$). The fixed-effect pooled estimation for TTF showed comparable results (HR = 1.35, 95% CI: 1.12-1.64, $P = 0.002$, Figure 3), suggesting that the outcome is significantly better in patients with advanced gastric cancer after CPT-11-containing combination chemotherapy than in those after non CPT-11-containing combination chemotherapy

Overall response rate

Risk ratio (RR) was reported in the 4 trials[18-21]. The overall response rate (ORR) of patients with advanced gastric cancer was 13%-80% to CPT-11-containing combination che-

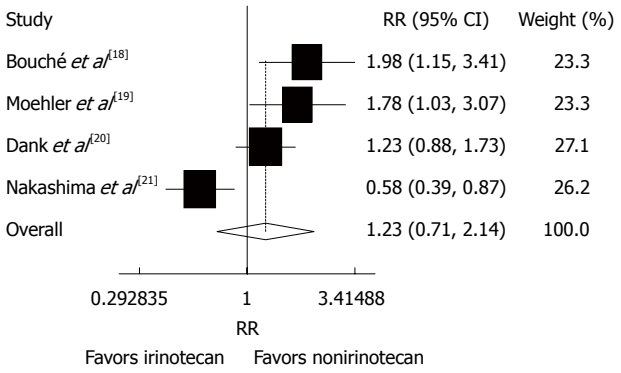

Figure 4 Overall response rate of patients with advanced gastric cancer after irinotecan-containing and nonirinotecan-containing combination chemotherapies. RR: Risk ratio.

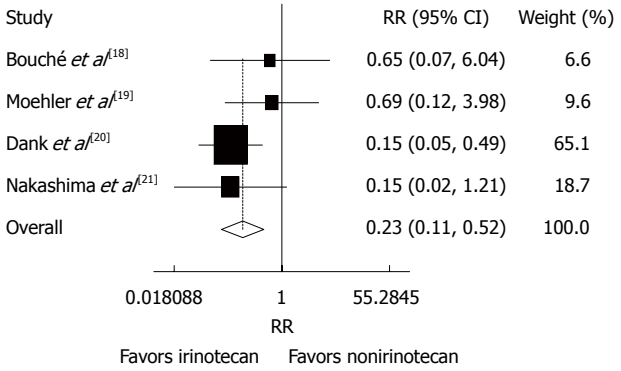

Figure 5 Grade 3/4 haematological toxicities (thrombocytopenia) in patients with advanced gastric cancer after irinotecan-containing and nonirinotecan-containing combination chemotherapies. RR: Risk ratio.

motherapy and 22%-47% to the non CPT-11-containing combination chemotherapy. A significant heterogeneity was observed ($P = 0.000$, $I^2 = 83.3\%$). The random-effect pooled estimate including 657 patients evaluated for ORR showed that the RR of CPT-11-containing combination chemotherapy was increased (RR = 1.23, 95% CI: 0.71-2.14, $P = 0.458$, Figure 4).

Toxicities

Reported toxicities were analyzed in all trials. The incidence of grade 3/4 thrombocytopenia (RR = 0.23, 95% CI: 0.11-0.52, $P < 0.001$) and diarrhea (RR = 4.00, 95% CI: 2.38-6.71, $P < 0.001$) was lower in patients after CPT-11-containing combination chemotherapy than in those after non CPT-11-containing combination chemotherapy (Figures 5 and 6). The incidence of other grade 3/4 haematological toxicities, such as neutropenia (RR = 0.60, 95% CI = 0.28-1.31, $P = 0.201$), febrile neutropenia (RR = 0.64, 95% CI: 0.37-1.10, $P = 0.108$) and leucopenia (RR = 0.85, 95% CI: 0.59-1.23, $P = 0.388$), as well as other grade 3/4 gastrointestinal toxicities, such as nausea (RR = 0.84, 95% CI: 0.46-1.54, $P = 0.582$), vomiting (RR = 0.83, 95% CI: 0.44 -1.56, $P = 0.556$), anorexia (RR = 0.66, 95% CI: 0.31-1.41, $P = 0.278$) was similar in patients with advanced gastric cancer after CPT-11-containing and non CPT-11-containing combination therapies. No evidence for heterogeneity was found except for neutropenia and alopecia.

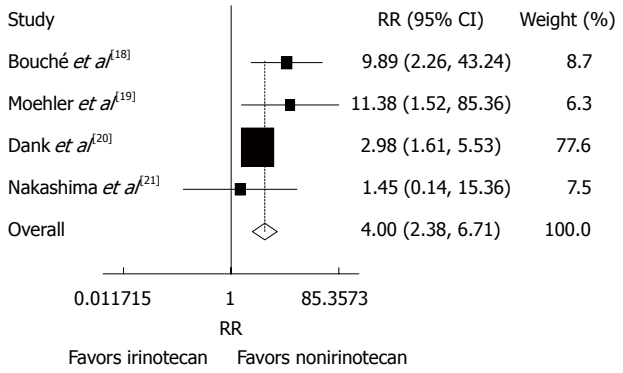

Figure 6 Grade 3/4 gastrointestinal toxicities (diarrhea) in patients with advanced gastric cancer after irinotecan-containing and nonirinotecan-containing combination chemotherapies. RR: Risk ratio.

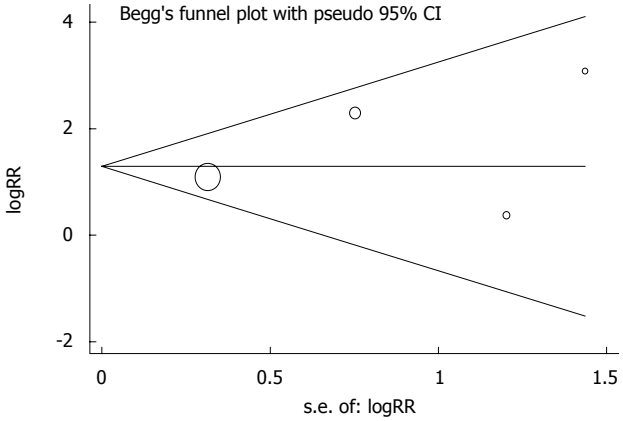

Figure 7 Funnel plots and Egger's linear regression test showing the potential publication bias in diagnosis of advanced gastric cancer[22]. RR: Risk ratio.

Publication bias

Evidence of publication bias was detected by plotting funnel plots of HR. Studies were plotted in order of decreasing variance of the log HR. Visual inspection of the funnel plots with respect to the 3 end points (OS rate, TTF and ORR) did not reveal any hint of publication bias (Figure 7). Funnel plots for all comparisons could not identify relevant publication bias, although the number of included studies was relatively small.

DISCUSSION

During the last decade, advances in treatment of patients with advanced gastric cancer have been achieved as a result of the integration of novel, effective agents into treatment algorithms. The availability of CPT-11 further enriches the options for combination therapy, because CPT-11 is effective and tolerable in patients with advanced gastric cancer.

The present systematic review revealed the major findings in ongoing debatable questions. We wonder whether

CPT-11-containing combination chemotherapy is better than non CPT-11-containing combination chemotherapy for advanced gastric cancer. One of the earlier systematic reviews on 3 randomized controlled trials (RCTs) of chemotherapy for advanced gastric cancer concluded that there is no convincing evidence that demonstrates a significant benefit in overall survival rate of patients after CPT-11-containing combination chemotherapy[27]. The resulting HR for the overall survival rate was 0.88% (95% CI: 0.73-1.06), which is in favor of CPT-11-containing combination chemotherapy. However, the study[23] did not assess other outcomes or toxicities. In our analysis, the 2 trials that assessed TTF showed that the overall summary estimate favored the CPT-11-containing combination chemotherapy with no significant inter-trial heterogeneity.

Most reported end points covered in the RCTs were searched and the most appropriate statistical methods for meta-analysis of time-to-event data extracted from published reports were used in our study. However, the quality of life was not stressed in patients with advanced gastric cancer due to the different methods used in reporting their quality of life. Although the data about response rate and adverse events were pooled to permit a clinically relevant analysis, these parameters varied. The response rate was reported according to the clinical parameters, WHO and RECIST criteria, whereas the CTC, WHO and ECOG scales were used in analysis of toxicity data.

In conclusion, there is insufficient evidence that the overall survival rate, overall response rate are better for patients with advanced gastric cancer after CPT-11-containing combination chemotherapy than after non CPT-11-containing combination chemotherapy. CPT-11-containing combination chemotherapy is advantageous over non CPT-11-containing combination chemotherapy for TTF and grade 3/4 thrombocytopenia.

Irrespective of the positive impact of presently available chemotherapy, the prognosis of patients with advanced gastric cancer remains poor, with a median survival time of 7-10 mo. Further RCTs are needed to assess which CPT-11 combination chemotherapy is least toxic.

COMMENTS

Background

The morbidity and mortality of gastric cancer increase with age. It has been shown that irinotecan (CPT-11) actis as a single agent in oesophago-gastric cancer. The aim of this meta-analysis was to assess the efficiency and toxicities of CPT-11 involved regimens in patients with advanced gastric cancer.

Research frontiers

Several phases Ⅱ and Ⅲ randomized trials have been reported comparing CPT-11-containing or non CPT-11-containing combination chemotherapy for advanced gastric cancer. The authors wonder whether CPT-11-containing combination chemotherapy is better than non CPT-11-containing combination chemotherapy for advanced gastric cancer.

Innovations and breakthroughs

This systematic review revealed the major findings in ongoing debatable questions. One of the earlier systematic reviews of 3 randomized controlled trials of chemotherapy for advanced gastric cancer concluded that there is no convincing evidence that demonstrates that the overall survival rate of patients with advanced gastric cancer is higher after CPT-11-containing combination chemotherapy than after non CPT-11-containing combination chemotherapy. However, this study did not assess other outcomes or toxicities. In the authors' analyses, 4 randomized phases Ⅱ and Ⅲ trials were included. A total of 657 patients were analyzed for their overall response rate, time to treatment failure (TTF), overall survival rate and toxicities. The two trials that assessed TTF showed that the overall summary estimate favored CPT-11-containing combination chemotherapy.

Applications

The findings in the study suggest that CPT-11 based combination chemotherapy is a candidate regimen for advanced gastric cancer. Further studies are needed to compare common chemotherapy with/without target therap.

Peer review

The authors in their meta-analysis showed that CPT-11-containing combination chemotherapy was advantageous over non CPT-11-containing combination chemotherapy for TTF, overall survival rate, overall response rate and toxicity. The availability of CPT-11 further enriches the options for combination therapy, because CPT-11 is effective and tolerable in patients with advanced gastric cancer. Further randomized control trials are needed to assess which chemotherapy provides favorable overall survival rate with less toxicity. Moreover, target therapy agents should be taken into consideration to see if they can achieve better clinical benefits in patients with advanced gastric cancer.

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BRIEF ARTICLE

Hepatic blood inflow occlusion without hemihepatic artery control in treatment of hepatocellular carcinoma

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Abstract

AIM: To investigate the clinical significance of hepatic blood inflow occlusion without hemihepatic artery control (BIOwHAC) in the treatment of hepatocellular carcinoma (HCC).

METHODS: Fifty-nine patients with HCC were divided into 3 groups based on the technique used for achieving hepatic vascular occlusion: group 1, vascular occlusion was achieved by the Pringle maneuver (n = 20); group 2, by hemihepatic vascular occlusion (HVO) (n = 20); and group 3, by BIOwHAC (n = 19). We compared the procedures among the three groups in term of operation time, intraoperative bleeding, postoperative liver function, postoperative complications, and length of hospital stay.

RESULTS: There were no statistically significant differences (P > 0.05) in age, sex, pathological diagnosis, preoperative Child's disease grade, hepatic function, and tumor size among the three groups. No intraoperative complications or deaths occurrred, and there were no significant intergroup differences (P > 0.05) in intraoperative bleeding, hepatic function change 3 and 7 d after operation, the incidence of complications, and length of hospital stay. BIOwHAC and Pringle maneuver required a significantly shorter operation time than HVO; the difference in the serum alanine aminotransferase or aspartate aminotransferase levels before and 1 d after operation was more significant in the BIOwHAC and HVO groups than in the Pringle maneuver group (P < 0.05).

CONCLUSION: BIOwHAC is convenient and safe; this technique causes slight hepatic ischemia-reperfusion injury similar to HVO.

Key words: Hepatic blood inflow occlusion without hemihepatic artery control; Hepatocellular carcinoma; Intraoperative bleeding; Ischemia-reperfusion injury

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INTRODUCTION

Intraoperative bleeding occurs most frequently during hepatic resection. Bleeding-associated blood transfusions increase the postoperative complications and mortality rate[1], especially in cases complicated with hepatocirrho-

sis[2]. Therefore, control of intraoperative bleeding is a prerequisite for reducing the number of cases requiring blood transfusions during liver resection. The common approaches to reduce blood transfusion include lowering the intraoperative central venous pressure[3-5], and using cut-ultrasound aspiration and microwave thermocoagulation in liver surgery[6,7], and hepatic inflow occlusion with or without outflow control. Hepatic inflow control (with or without outflow control) plays an important role in constructing a bloodless surgical field. However, hepatic ischemia-reperfusion injury (HIRI) occurs in all hepatic vascular occlusions to some extent. The normal liver receives 70%-75% of its blood supply from the portal vein and 40%-60% of its oxygen supply from arterial blood. Considering the differences between the distribution and oxygen content of the blood in the portal vein and that in the hepatic artery, we performed hepatic blood inflow occlusion without hemihepatic artery control (BIOwHAC)[8] to minimize HIRI by a modified surgical procedure. During BIOwHAC, the proper hepatic artery was surgically exposed, after which its left and right branches were separated. For the right and left hemihepatic occlusions, catheters were advanced and bypassed the portal vein, bile duct, and the respective branches of the hepatic artery before being tightened.

We designed a retrospective case-control study to compare BIOwHAC, Pringle maneuver and hemihepatic vascular occlusion (HVO) in terms of operation time, intraoperative bleeding, and postoperative liver function, and postoperative complications. We also assessed the merits and demerits of these three approaches.

MATERIALS AND METHODS

Inclusion criteria

Between March 2005 and January 2009, 162 patients were treated at our hospital for liver neoplasms. Of these, 59 patients who met with the following criteria were included in this trial: availability of complete data, presence of pathologically diagnosed hepatocellular carcinoma (HCC), cancers confined to half of the liver and being suitable for hepatic portal anatomical vascular occlusion, and resectable tumors. Patients who underwent pericardial devascularization, splenectomy, gastroenterostomy and biliointestinal anastomosis were excluded.

Vascular occlusion procedures

Of the 59 patients, 20 underwent Pringle maneuver, 20 underwent HVO, and 19 underwent BIOwHAC. The patients in the Pringle maneuver group underwent the Pringle's occlusion by the conventional method at the hepatoduodenal ligament using urethral catheters. Patients in the HVO group underwent vascular occlusions *via* two approaches. (1) The catheters were introduced into the confluence of the left and right hepatic ducts along the common hepatic duct without dissecting the hepatoduodenal ligament; the tangential clamp was placed at the superior hepatic capsule or inferior transverse fissura ligamenti teretis and then the internal liver parenchyma and the external Glisson sheath were bluntly separated with care; if no resistance occurred at that time, the urethral catheter was exited at the portal vein bifurcation-caudate lobe junction towards the posterior hepatoduodenal ligament and then insufflated to achieve right hemihepatic occlusion; one end of the catheter was inserted through the lesser omentum foramen to the hepatogastric ligament and insufflated to achieve left hemihepatic occlusion; and (2) The affected side of the hepatic artery and the portal vein were separated to occlude them simultaneously or individually. Patients in the BIOwHAC group underwent BIOwHAC as follows: the proper hepatic artery was surgically exposed and the left and right branches of the proper hepatic artery were separated; for right hemihepatic occlusion, the catheter bypassed the portal vein, bile duct, and the right hepatic artery and was tightened; for left hemihepatic occlusion, the catheter bypassed the portal vein, bile duct, and the left hepatic artery and was tightened (Figure 1).

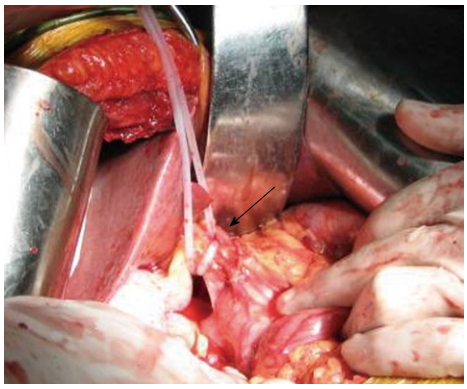

Figure 1 Application of hepatic blood inflow occlusion without hemihepatic artery control (arrow shows non-occlusive left hepatic artery).

Data collection

Complete background information, including age, sex, liver function, Child's disease grade, level of serum markers of hepatitis, and α-fetoprotein levels was collected from all the patients.

Computed tomography (CT) and magnetic resonance imaging (MRI) images and operative record were carefully previewed and information regarding the size of HCC, hepatic vascular occlusion approach, occlusion time, type of liver resection, and operation time were precisely recorded.

The levels of albumin, total bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were recorded 1, 3, and 7 d after operation to determine the deviations from preoperative values.

Patients were observed for postoperative complications such as bleeding from hepatic section, biliary fistula, subphrenic abscess, responsive pleural effusion, hepatic encephalopathy, pulmonary infection, hemorrhage from

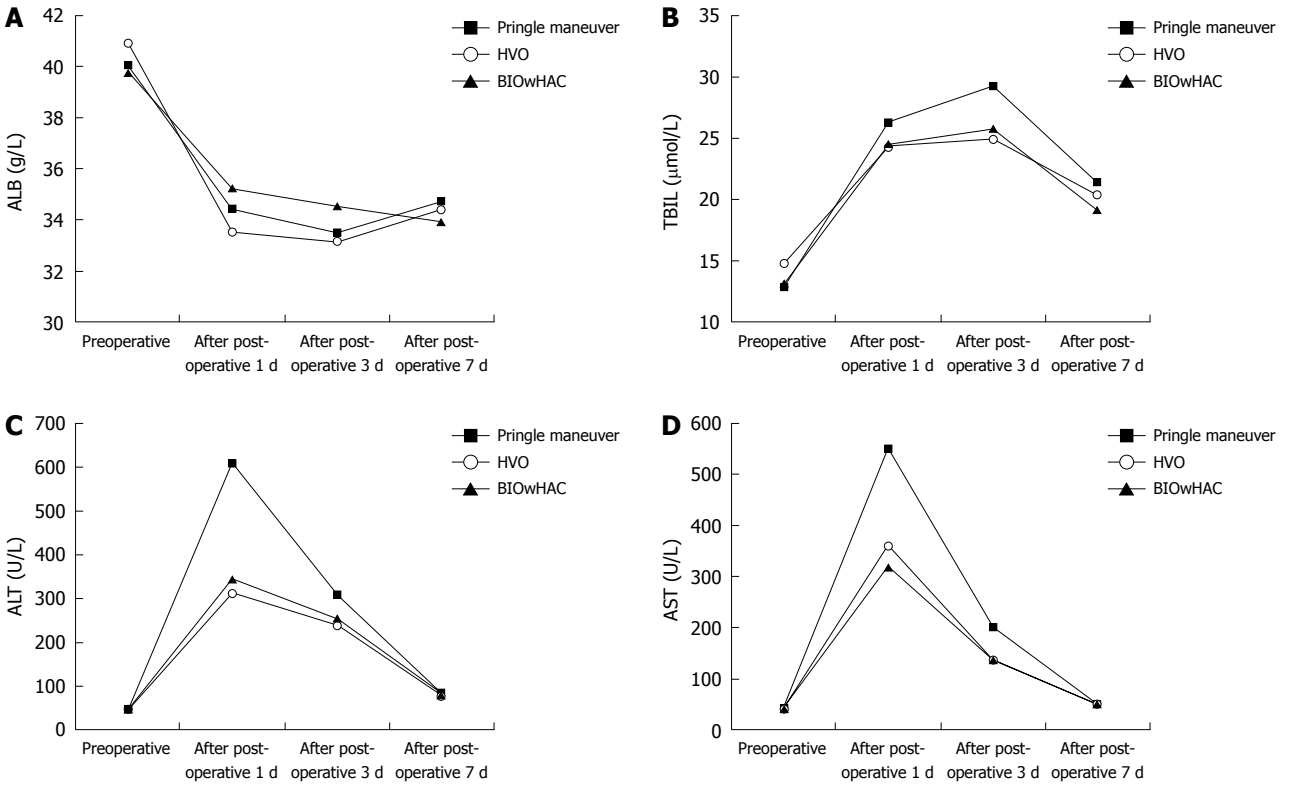

Figure 2 Preoperative and postoperative albumin concentrations (A), total bilirubin levels (B), serum alanine aminotransferase levels (C) and aspartate aminotransferase levels (D) in the 3 approaches for achieving occlusions. HVO: Hemihepatic vascular occlusion; BIOwHAC: Hepatic blood inflow occlusion without hemihepatic artery control; ALB: Albumin; TBIL: Total bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Table 1 Patients' background information[1] (mean ± SD)

Groups	Pringle maneuver (n = 20)	HVO (n = 20)	BIOwHAC (n = 19)
Age (yr)	54.35 ± 10.03	54.20 ± 11.34	54.26 ± 7.05
Gender (M:F)	16:4	13:7	14:5
Pathologic diagnosis	HCC	HCC	HCC
Complication hepatocirrhosis (cases)	10	9	10
Hepatitis virus positive (cases)	18	18	17
Increase in AFP (cases)	12	11	10
Child's disease grade (cases, A:B)	19:1	19:1	18:1
Serum albumin (g/L)	40.04 ± 3.42	40.87 ± 3.12	39.78 ± 2.71
Serum bilirubin (μmol/L)	12.86 ± 5.60	14.68 ± 3.37	13.08 ± 7.81
Serum ALT (U/L)	45.22 ± 32.99	45.04 ± 63.01	47.63 ± 40.48
Serum AST (U/L)	39.43 ± 17.20	40.72 ± 21.57	41.56 ± 41.01

[1]There is no significant intergroup difference (P > 0.05). BIOwHAC: Hepatic blood inflow occlusion without hemihepatic artery control; HVO: Hemihepatic vascular occlusion; AFP: α-fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

the digestive tract, and long-term (> 30 d) hepatic dysfunction.

Statistical analysis

Data were expressed as mean ± SD and variance analysis was performed; χ^2 test was performed for countable data. P < 0.05 indicates significant difference.

RESULTS

Background information

Patients' information on age, sex, preoperative Child's disease grade, pathologic diagnosis, and preoperative hepatic function are listed in Table 1; and the information about tumor size and type of liver resection is shown in Table 2.

Intraoperative information

In all the patients, the procedures were performed without intraoperative complications and deaths. Each group underwent vascular occlusion only once, and there were no statistically significant intergroup differences (P > 0.05) in occlusion time, type of liver resection, bleeding and blood transfusions (Table 2). BIOwHAC and Pringle maneuver required significantly shorter operation time than HVO (151.84 ± 41.77 min and 158.50 ± 43.77 min, respectively *vs* 219.25 ± 58.09 min, P < 0.05), but the time required for BIOwHAC was almost equivalent to that for Pringle maneuver (P > 0.05).

Postoperative hepatic function variation

Serum albumin level was reduced in all the patients 1 and 3 d after operation, but increased after 7 d, and no significant intergroup difference was observed (Figure 2A). The total serum bilirubin level increased 1 and 3 d after operation, but decreased after 7 d, with no significant intergroup differences (Figure 2B). In addition, there was an increase in serum ALT and AST levels 1 d after operation

Table 2 Intraoperative status of patients (mean ± SD)

Groups	Pringle maneuver (n = 20)	HVO (n = 20)	BIOwHAC (n = 19)
Right hemiliver (cases)	2	3	1
Left hemiliver (cases)	1	2	1
S_2 + S_3 (cases)	3	5	3
S_6 + S_7 (cases)	2	2	2
S_5 + S_6 (cases)	2	2	1
S_5 + S_8 (cases)	1	2	1
S_6 (cases)	2	2	3
S_7 (cases)	3	1	2
S_5 (cases)	2	1	2
S_4 (cases)	2	0	2
S_8 (cases)	0	0	2
Tumor size (cm)	6.33 ± 3.39	7.60 ± 4.03[b]	6.11 ± 3.18[b,c]
Operation time (min)	158.50 ± 43.77[a]	219.25 ± 58.09	151.84 ± 41.77[a]
Occlusion time (min)	20.60 ± 4.91	25.70 ± 8.29[b]	18.94 ± 5.13[b,c]
Occlusion frequency (time)	1	1	1
Bleeding volumes (mL)	700.00 ± 163.32	1017.5 ± 663.57[b]	789.47 ± 683.04[b,c]
Blood transfusion (cases)	8	11	9
Transfusion volumes (mL)	775.00 ± 679.81	709.09 ± 317.66[b]	766.66 ± 580.94[b,c]

[a]P < 0.05 *vs* HVO; [b]P > 0.05 *vs* pringle maneuver; [c]P > 0.05 *vs* HVO. BIOwHAC: Hepatic blood inflow occlusion without hemihepatic artery control; HVO: Hemihepatic vascular occlusion.

Table 3 Postoperative complications and length of hospital stay (mean ± SD)

Groups	Pringle maneuver (n = 20)	HVO (n = 20)	BIOwHAC (n = 19)
Postoperative complications (cases)	4	5	4
Responsive pleural effusion (cases)	3	2	3
Pulmonary infection (cases)	1	3	1
Hepatic section bleeding (cases)	0	0	0
Bile leakage (cases)	0	0	0
Subphrenic abscess (cases)	0	0	0
Hepatic encephalopathy (cases)	0	0	0
Hemorrhage of digestive tract (cases)	0	0	0
Long-term liver dysfunction (cases)	0	0	0
Perioperative deaths (cases)	0	0	0
Length of hospital stay (d)	21.75 ± 4.32	22.95 ± 5.30	21.47 ± 9.36

BIOwHAC: Hepatic blood inflow occlusion without hemihepatic artery control; HVO: Hemihepatic vascular occlusion

but a decrease after 7 d. Patients in the Pringle maneuver group had more significant variation in the serum ALT and AST levels (P < 0.05) before and 1 d after operation than the BIOwHAC and HVO groups, but there was no significant difference between BIOwHAC and HVO groups (P > 0.05). No significant intergroup differences were noted in the serum ALT and AST levels (P > 0.05) 3 and 7 d after operation (Figure 2C and D).

Postoperative complications and length of hospital stay

The most common postoperative complication in this study was pleural effusion with pulmonary infection, occurring in 4 cases (20%) of the Pringle maneuver group, 5 cases (25%) of the HVO group, and 4 cases (21.1%) of the BIOwHAC group. No significant intergroup differences were noted in length of hospital stay among the three groups (Table 3).

DISCUSSION

Owing to the recent improvement in the liver resection technique intraoperative hepatic vascular occlusion and perioperative management, surgical complications and mortality rate have been considerably reduced[8-10]. Pringle maneuver is the commonly used method for hepatic vascular occlusion, which enables effective control of bleeding. Multiple approaches for hepatic vascular occlusions have been developed; however the Pringle maneuver is more popular because it can be used in various types of liver resection[11,12]. Man *et al*[13] reported that the Pringle maneuver is superior to non-vascular occlusion, but can lead to obvious HIRI and is time consuming, especially in cases complicated with liver cirrhosis[14]. In 1987, Makuuchi performed HVOs through portal venous branch occlusion and hepatic arterial branch occlusion[15]. HVO helps conserve the contralateral hepatic vascular inflow and facilitates the hepatic operation and mildly affects postoperative liver function in favor of patients with liver cancer and hepatocirrhosis[15]. However, HVO must be performed by surgeons who are proficient in portal vein surgery so as to avoid incident damage to the interior conduit of the Glisson sheath, hemorrhage, and biliary fistula. If there are communicating branches between the non-occluded and the occluded hemilivers, hepatic bleeding may be quite severe[16].

Although no significant intergroup differences were observed in the changes of the serum AST and ALT levels 3 and 7 d after operation, patients in the HVO and BIOwHAC groups had significantly lower AST and ALT levels than those in the Pringle maneuver group 1 d after

operation. This implies that HVO and BIOwHAC are superior to Pringle maneuver in minimizing the HIRI. In HVO, the vascular occlusion is continuous; but in the Pringle maneuver the circulation is maintained with occlusion for 15-20 min and reperfusion for 5 min, this interrupted occlusion gives rise to several problems[3]. In our study, the patients underwent only a single vascular occlusion, which is the reason why no significant intergroup differences were seen in intraoperative bleeding and in the number of cases requiring blood transfusions.

In humans, the confluence of the left and right hepatic ducts lies superiorly, the portal bifurcation is inferior to this bifurcation, and below is the proper hepatic arterial bifurcation. Therefore, it is convenient and safe to intraoperatively expose the proper hepatic artery and separate its left and right branches. Thus, surgeons can occlude the portal vein, bile duct, and occlusive-side artery branch in a single operation. BIOwHAC is convenient and safe as compared to Pringle maneuver. Our results showed that HVO required significantly longer operation time than BIOwHAC and Pringle maneuver (219.25 ± 58.09 min *vs* 151.84 ± 41.77 min and 158.50 ± 43.77 min) due to the net operation of vascular occlusion for 20-30 min. In addition, resection of the cholecyst is required in the right HVO, but not in BIOwHAC, which can satisfy the wish of the patients who want to conserve the cholecyst.

In the normal liver, 70%-75% of blood supply comes from the portal vein and 40%-60% of oxygen supply from arterial blood. Portal vein can not supply all parts of the liver with oxygen, and when the portal vein is completely occluded, the level of oxygen consumption in the liver remains the same as that before occlusion. This indicates that the hepatic artery alone is sufficient to meet the oxygen demand of the liver[17]. Our results showed that the patients in the BIOwHAC group had significantly lower serum AST and ALT levels than those in the Pringle maneuver group 1 d after operation, but no significant differences from that in the HVO group. This finding shows that hemihepatic artery conservation can provide enough oxygen to meet the demand of the intact hemiliver. In addition, no significant intergroup difference was noted in the postoperative serum albumin levels, which may be attributed to postoperative exogenous supplements.

Our results indicated no significant intergroup differences in the intraoperative bleeding and blood transfusion, liver function 3 and 7 d after operation, incidence of postoperative complications, and length of hospital stay. We recommend BIOwHAC because it is a convenient and safe technique similar to the Pringle maneuver and can protect the liver function injury as effectively as HVO.

In conclusion, among the various kinds of approaches for hepatic vascular occlusion available currently, the most suitable one should be selected on the basis of comprehensive preoperative examinations (CT or MRI imaging and liver function tests), intraoperative examinations (pathological examination and lesion location), examinations for invasion in the hepatic vein and inferior vena cava, cardiovascular status, as well as the experience and skill of surgeons and anesthesiologists.

COMMENTS

Background

Intraoperative bleeding occurs most commonly during hepatic resection. Hepatic inflow control plays an important role in constructing a bloodless surgical field.

Research frontiers

There are various techniques of hepatic vascular control, including Pringle maneuver, hemihepatic vascular occlusion (HVO), total hepatic vascular exclusion, liver hanging maneuver, *etc*.

Innovations and breakthroughs

Based on the differences between the distribution and oxygen content of the blood in the portal vein and that in the hepatic artery, hepatic blood inflow occlusion without hemihepatic artery control (BIOwHAC) was performed in this study.

Applications

BIOwHAC is convenient and safe similar to Pringle maneuver; this technique causes as slight hepatic ischemia reperfusion injury as HVO.

Terminology

BIOwHAC: the proper hepatic artery was surgically exposed, after which its left and right branches were separated. For the right and left hemihepatic occlusions, catheters were advanced and bypassed the portal vein, bile duct, and the respective branches of the hepatic artery before being tightened.

Peer review

This is a retrospective analysis on a single center series of different methods of inflow hepatic occlusion during liver resection for liver cancer. The study is well written and the methodology is correct.

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BRIEF ARTICLE

Human papillomavirus DNA and P16^{INK4A} expression in concurrent esophageal and gastric cardia cancers

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Abstract

AIM: To investigate the relationship between human papillomavirus (HPV) infection and concurrent esophagus and gastric cardia cancer from the same patient (CC) and examine the significance of P16^{INK4A} protein expression.

METHODS: Polymerase chain reaction was used to detect the presence of HPV type16 (HPV16). The expression of P16^{INK4A} protein was detected using immunohistochemistry.

RESULTS: Among the CC specimens, HPV16-DNA was found in eight cases of esophageal squamous cell carcinoma (ESCC) and five cases of gastric cardia adenocarcinoma (GCA), respectively (47% *vs* 29%), and two of both ESCC and GCA. P16^{INK4A} was highly expressed in both ESCC and GCA. In the HPV-associated positive CC, higher P16^{INK4A} expression was observed in the GCA than in the ESCC (75% *vs* 25%, $P < 0.05$).

CONCLUSION: HPV16 as a correlated risk factor may play an important role in the development of ESCC and GCA. P16^{INK4A} may be a screening index in the HPV-associated carcinoma of gastric cardia.

Key words: Esophageal squamous cell carcinoma; Gastric cardia adenocarcinoma; Human papillomavirus; Polymerase chain reaction; Immunohistochemistry

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INTRODUCTION

Esophageal carcinoma (EC) is one of the most common

malignant diseases worldwide. Linzhou (formerly known as Linxian) in Henan Province, northern China, has been recognized as the highest prevalence area of esophageal squamous cell carcinoma (ESCC) in the world[1]. In epidemiology, gastric cardia adenocarcinoma (GCA) in China is characterized by its striking geographic distribution and its concurrence with ESCC[2]. Similar phenomenon can be also observed in other esophageal cancer incidence areas worldwide[3]. At present, ESCC and GCA still are the main cause of tumor-related deaths in this area. Another interesting feature at the high prevalence area is that the primary cancers of the esophagus and gastric cardia are concurrent in the same patient in Henan, we named them concurrent carcinoma of the esophagus and gastric cardia in the same patient (CC), which is not uncommon in this area (0.4%-2.5%)[4]. The special pattern suggested that similar risk factors and mechanism might be involved in these two cancers.

Human papillomavirus (HPV) as one of the important tumor-related viruses has been firmly recognized in cervical cancer with HPV-DNA detected in > 99% specimens[5]. However, its oncogenic role in other tumors still remains controversial. As to its role in ESCC, it was firstly mentioned by Syrijanen twenty years ago[6]. Since then, many reports regarding this topic have been published, but the HPV infection rate in ESCC varied from zero to 90%[7,8] and in GCA from zero to 68%[9,10], depending on the specimens obtained from low- or high-risk areas and the methods used in each study[11]. In order to further investigate the prevalence of HPV infection in upper digestive tract tumor, the samples with concurrent ESCC and GCA from the same patient were tested for the existence of HPV type16 (HPV16)-DNA.

HPVs are small DNA viruses that can be classified as either high-risk or low-risk types. HPV-16 and 18 is most common in high-risk group, especially type 16 which is considered a risk factor for EC at a high prevalence area in Henan[8,12]. The E6 and E7 oncoproteins of the high-risk HPV types can efficiently destroy the cell cycle regulation and apoptotic pathways by binding to a number of host cell proteins, such as $P16^{INK4A}$ protein, which is an inhibitor of cyclin-dependent kinase. The HPV oncoproteins are able to alter the cell cycle and leaves them vulnerable to other genetic changes, ultimately resulting in malignant transformation[13,14]. However, the reports about HPV infection involved in the carcinogenesis of gastric cardia are very limited, and there has been no related study comparing the HPV detection rate and expression of $P16^{INK4A}$ protein in CC tissues. In our study, we investigated the HPV infection and changes of $P16^{INK4A}$ protein in the same concurrent cancer patient in the high-risk area of EC in Henan to understand the mechanism of esophagus/cardia carcinogenesis in this area, and further illustrate whether the two carcinomas have the similar pathogenesis.

MATERIALS AND METHODS

Clinical samples and diagnostic criteria of CC

A total of 23 cases of concurrent ESCC and GCA were obtained. Among them, DNAs from 17 ESCC and their corresponding GCA tissues were extracted from paraffin-embedded samples by conventional phenol-chloroform procedure. Six cases were from Linzhou Center Hospital, eight from Linzhou Yaocun Esophageal Cancer Hospital and three from Anyang City Cancer Hospital from September 2005 to June 2008. All the hospitals are located in the high incidence region of ESCC in Henan. There were twelve men and five women with an average age of 58 years. None of the patients received chemo- and radiotherapy before surgery.

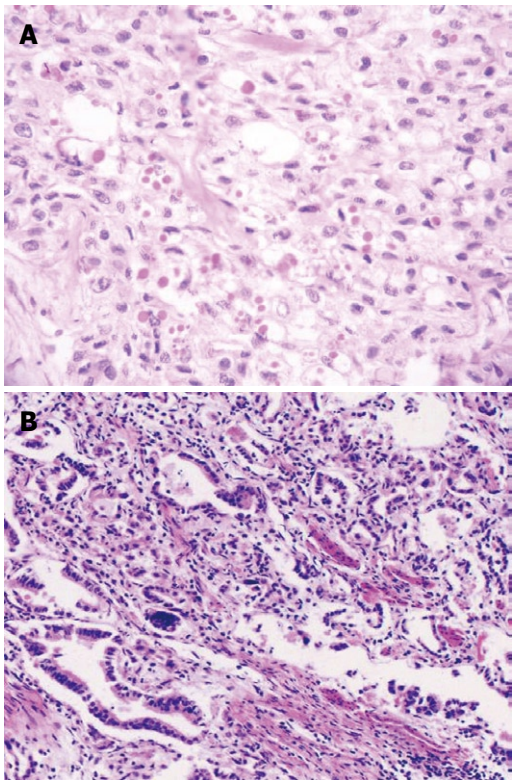

Figure 1 Hematoxylin and eosin staining in the concurrent esophageal and gastric cardia cancers tissue specimens. A: Esophageal squamous cell carcinoma tissue (× 100); B: Gastric cardia adenocarcinoma tissue (× 100).

The samples were immediately fixed by 10% formalin, dehydrated and paraffin-embedded, followed by pathological diagnosis and immunochemical analysis. The diagnosis of the CC was based on the following criteria: (1) All the tumors in the esophagus and cardia in the same patient are malignant; (2) All the tumors have defined pathological modality, i.e. concurrent esophageal squamous cell carcinomas and gastric cardia adenocarcinomas; and (3) None the tumors are metastatic (Figure 1). This study was approved by the Institutional Review Board of the School of Medicine, Zhengzhou University, China.

DNA extraction

The methods were used as described by Greer *et al*[15]. Briefly, each formalin-fixed and paraffin-embedded sample was cut into 10 μm thick sections, 5-10 slides were deparaffinized in xylene and graded alcohol, then the lysis buffer (300 mmol/L NaCl; 50 mmol/L Tris HCl pH 8.0; 0.2% SDS) was added into the tube with proteinase K

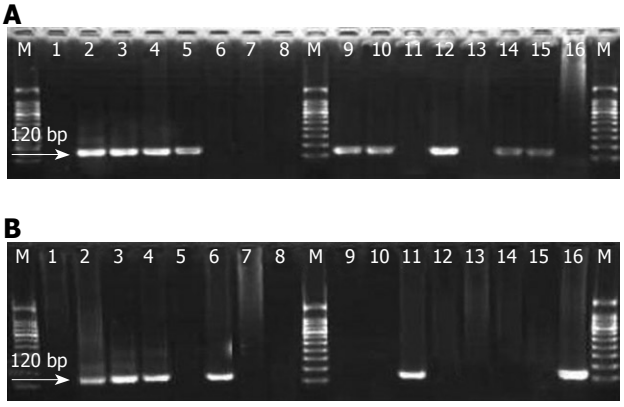

Figure 2 Amplification of human papillomavirus type 16-E6 gene fragment in the concurrent esophageal and gastric cardia cancers tissues. Polymerase chain reaction products were run in 3.0% agarose gel; Lane M: Molecular marker (100 bp ladder); Lane 1: Double water (negative control); Lane 2: Plasmid with human papillomavirus type 16 (HPV16)-E6 120 bp (positive control); Lanes 3-16: Represent positive and negative cases. A: HPV16-E6 gene fragment amplification in esophageal squamous cell carcinoma tissues; B: HPV16-E6 gene fragment in the corresponding gastric cardia adenocarcinoma tissue amplification.

(200 mg/L), and the solution was incubated at 55℃ overnight until it became clear. DNA was then extracted using conventional phenol-chloroform procedure, precipitated with cold alcohol and dissolved in ion-free water, and the concentration was determined based on its optical density. Quality of the extracted DNA was tested by polymerase chain reaction (PCR) with β-actin primer: 5'-TCACCCACACTGTGCCCATC-3' and 5'-GAACCGCTCATTGCCAATGG-3'. The DNA which was β-actin gene amplification positive was used to detect the presence of HPV16-DNA.

PCR and P16^{INK4A} protein immunostaining

The usable DNA went through PCR amplification using type-specific primer: 5'-TCAAAAGCCACTGTGTCCTG-3' and 5'-CGTGTTCTTGATGATCTGCA-3' targeting HPV16-E6 gene. Recombinant plasmid DNA HPV16$^{-pBR322}$ as positive control was obtained from Professor You-Lin Qiao, the Cancer Research Institute, Chinese Academy of Medical Sciences and double water was used as negative control in PCR. Reactions were set in 25 μL 1 × PCR buffer containing 10 mmol/L Tris HCl, pH8.4, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 4 mmol/L dNTP and 1.5 U Taq DNA polymerase (Promega). PCR conditions were as follows: an initial denaturation at 95℃ for 5 min followed by 5 cycles with cycling profile at 95℃ denaturing for 1 min, at 55℃ annealing for 1 min, and at 72℃ prolonging for 1 min, and then at 95℃ denaturing for 20 s, at 55℃ annealing for 30 s and at 72℃ prolonging for 30 s with 45 cycles[8]. The amplified products were revealed by electrophoresis on 3.0% agarose gels (Promega) containing 0.5% μg/mL of ethidium bromide in 1 × TAE buffer at 100 volts for 30 min. Samples were considered positive if a band of 120 bp was observed under the ultraviolet light (Figure 2). All the experiments from DNA extraction to HPV gene amplification were carried out at the Virus Research Institute, Chinese Academy of Preventive Medicine in Beijing, China by the same technician with the same protocol as established previously in Dr. Yi Zeng's laboratory. Positive control (plasmid with HPV16$^{-pBR322}$ DNA fragment) and negative control (double water) were set in each HPV-DNA amplification process. Each experimental step was taken following the previously established rules.

For immunohistochemistry, sections of paraffin-embedded tissue with a thickness of 3 μm were deparaffinized by passage through xylene. After the endogenous peroxidase activity was blocked with 0.3% H_2O_2, the slides were then rehydrated with 0.01 mol/L sodium phosphate/citrate buffer at pH 8.0 and heated in 0.01 mol/L-citrate buffer at pH 6.0, 95℃ for 30 min to retrieve the antigen. After rinsed in 0.01 mol/L phosphate-buffered saline (PBS) at pH 7.4, nonspecific antibody binding was reduced by incubating the sections with 10% fetal bovine serum in PBS for 30 min. The sections were incubated overnight at 4℃ with a mouse monoclonal antibody of p16^{INK4A} protein (1:200 dilution, PharMingen International). After washing thoroughly with PBS, the slides were incubated with biotinylated horse anti-mouse IgG for 30 min followed by 1:100 dilution of the Avidin-Biotin-Peroxidase Complex (Vectastain elite ABC kit, Vector Laboratories, Burlingame, CA) for an additional 30 min. The peroxidase signal was visualized by treatment with DAB substrate-chromogen system (DAKO) for 8 min. Finally, the sections were stained lightly with hematoxylin. In statistical analysis, those having less than 10% cells stained positive were classified as negative and the others were regarded as positive cases[16] (Figure 3).

Statistical analysis

Fisher's exact test was used to examine the association between HPV status and each clinicopathological factor including p16 expression. All the *P* values presented in the present study were two-sided. Experimental data were analyzed by statistical software SPSS 13.0.

RESULTS

The results of the PCR showed that, in the 17 CC, the detection rate of HPV16 in the ESCC was 47% (8/17), and 29% (5/17) in GCA. There was no significant difference between the two groups ($P > 0.05$). Two positive and six negative cases of ESCC and GCA were found in the HPV16-DNA simultaneously, illustrating the high consistency of HPV involved in the two kinds of tumors (47%) (Table 1).

In the 17 cases with CC, the positive ratio of P16^{INK4A} protein expression was 41% (7/17) in ESCC and 59% (10/17) in GCA, respectively. Twelve patients showed P16^{INK4A} protein changes (71%), among them, six patients were P16^{INK4A} immunoreaction positive (35%) in ESCC and GCA simultaneously.

According to the Fisher's Exact test, there was no significant difference between HPV infection and P16^{INK4A} protein expression in the ESCC and GCA specimens ($P > 0.05$). However, among the eight HPV positive ESCC

Table 1 Esophageal squamous cell carcinoma and gastric cardia adenocarcinoma in the same patient: Clinical and pathological features

Sample No.	Age (yr)	Gender	ESCC				GCA			
			Differentiation	Stage	HPV	P16	Differentiation	Stage	HPV	P16
1	57	Male	M	T1N0M0	+	-	M	T3N0M0	+	+
2	60	Male	M	T1N0M0	+	+	L	T2N0M0	+	+
3	62	Female	H	T3N0M0	+	-	M	T2N0M0	-	+
4	62	Male	M	T2N0M0	-	+	H	T2N0M0	+	-
5	50	Male	M	T1N0M0	-	+	M	T3N0M0	-	+
6	68	Male	M	T1N0M0	-	-	H	T3N0M0	-	-
7	71	Male	M	T3N0M0	+	-	L	T3N0M0	-	-
8	61	Female	M	T2N0M0	+	-	M	T1N1M0	-	+
9	51	Male	H	T3N0M0	-	+	L	T3N0M0	+	+
10	48	Male	M	T1N0M0	+	-	M	T3N0M0	-	-
11	57	Female	M	T3N0M0	-	-	L	T3N0M0	-	-
12	55	Male	H	T2N0M1	+	+	M	T3N1M0	-	+
13	57	Female	L	T3N0M0	+	-	M	T1N0M0	-	+
14	67	Male	M	T2N0M0	-	+	L	T1N0M0	+	+
15	64	Male	M	T3N0M0	-	+	L	T2N0M0	-	+
16	51	Male	M	T1N0M0	-	-	L	T3N1M0	-	+
17	52	Female	H	T1N0M0	-	-	L	T3N0M0	-	-

ESCC: Esophageal squamous cell carcinoma; GCA: Gastric cardia adenocarcinoma; HPV: Human papillomavirus; M: Moderately differentiated; H: Highly differentiated; L: Low differentiated.

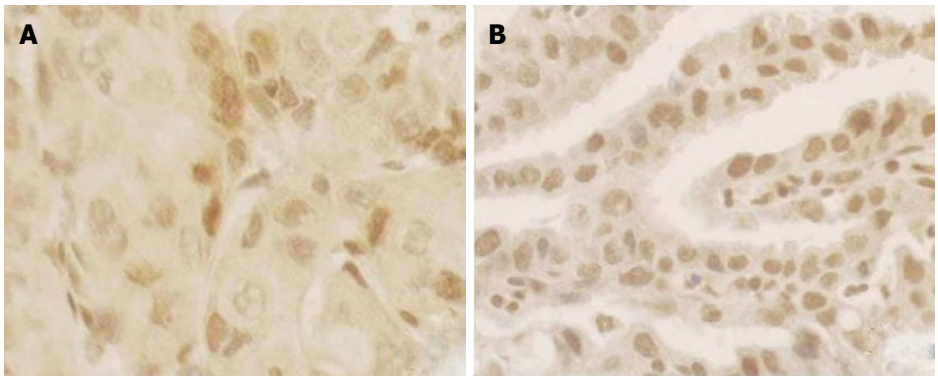

Figure 3 $P16^{INK4A}$ protein expression in the concurrent esophageal and gastric cardia cancers tissues by immunohistochemical staining Avidin-Biotin-Peroxidase Complex method (× 200). A: $P16^{INK4A}$ protein expression in esophageal squamous cell carcinoma; B: Expression of $P16^{INK4A}$ protein in gastric cardia adenocarcinoma.

specimens, only two expressed $P16^{INK4A}$, while four expressed $P16^{INK4A}$ in the HPV positive GCA tissues.

DISCUSSION

The present study demonstrates that both ESCC and GCA tissues from the same patient had HPV16-DNA infection, with an incidence of 47% and 29%, respectively. And HPV16 E6-DNA was observed in both ESCC and GCA tissues in two patients simultaneously. These results suggest that HPV16 might participate in the carcinogenesis of the ESCC and GCA.

In the Linzhou area in Henan, almost half of the ESCC patients have HPV16 infection, with a higher incidence than in GCA (47% *vs* 29%), but without a significant difference ($P > 0.05$). However, there were no correlations between HPV16 infection and gender, age, tumor size, depth of penetration, differentiation, lymph node metastasis and TNM stage (all $P > 0.05$). The difference and the mechanism of the affinity specificity of HPV16 to squamous epithelium and styloid glandular epithelium are still not clear[17]. Esophageal epithelium and cardia epithelium are under the same internal environment and heredity of the same organism, as well as exposure to the same environment and carcinogenic agents, which may be associated with the co-infection of the two different epithelial tissues for HPV. Recently, it has been reported that colonic epithelium and colon carcinoma have HPV infections[18-20], and further studies are still needed to confirm the biological significance and mechanisms for HPV invading the body.

Over the past 20 years, many reports regarding HPV infection in EC have been published, and the reported HPV detection rate in the literature varied largely. To explain these marked differences, different region, sampling methods, demographic and ethnic factor, disease status, and sensitivity of detection methods have been cited as potential causes of this inconsistency[11,21]. In the present

study, the same method was used to analyze the two kinds of neoplasms with different histological type in the same patient, which greatly reduced the disparity of the methodology and/or population.

CC is an ideal model for illustrating the environmental influence on both ESCC and GCA with the similar genobackground and comparability of environmental agents. The present study will deepen the understanding on the mechanism of esophageal/cardia carcinoma in this area. Although these results indicating the presence of HPV-DNA in esophagus and gastric cardia carcinoma tissues, suggest a possible role for HPV in upper digestive tract tumors, further studies are necessary for establishing a definite causative role.

$P16^{INK4A}$ gene is an important member of P53-Rb system, and its product $P16^{INK4A}$ protein can prevent the cell to enter S-phase from G1-phase, and suppress cell proliferation, through inhibition of the phosphorylation of the retinoblastoma (pRb)[22]. In this cascade regulation, pRb could negatively inhibit the expression of $P16^{INK4A}$ protein.

The studies of cervical cancer showed that the HPV-E6/E7 protein combined with pRb is deactivated, and removes the negative inhibition of the expression of $P16^{INK4A}$ protein, which causes the over-expression of $P16^{INK4A}$. In the present study, 7 (41%) cases showed the expression of $P16^{INK4A}$ protein in ESCC, and 10 (59%) in GCA, which is similar to the results in the adenocarcinoma of the uterine cervix by Ansari-Lari *et al*[23]. In the study of HPV-associated cervical cancer, expression of $P16^{INK4A}$ protein is higher in the adenocarcinoma than in the squamous cell carcinoma, and combined detection of HPV infection and $P16^{INK4A}$ protein expression would be helpful to the diagnosis of the primary adenocarcinoma of the uterine cervix[24]. In the present study, 2 of the 8 HPV-positive ESCC cases expressed $P16^{INK4A}$, while 4 of 5 HPV-positive GCA cases expressed $P16^{INK4A}$. These results suggested that in the HPV- associated CC, expression of $P16^{INK4A}$ protein in GCA is higher than in ESCC. It is obvious that it would be of great significance to further understand the molecular discrepancy of the HPV16 positive and negative patients, and explore the exact mechanisms of the role of HPV in the carcinogenesis of esophagus and cardia.

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COMMENTS

Background

Esophageal cancer (EC) is one of the most common malignant diseases, with a remarkable geographical distribution and poor prognosis. The five-year survival rate is only 10%. However, the five-year survival rate for the patients with the early EC is more than 90%. More than 85% of the EC patients are diagnosed at the late stage due to lack of early specific symptoms and unknown etiological factors, and the carcinogenesis remains the leading cause of late diagnosis for EC. Therefore, the current challenges in EC research are to obtain a better understanding of the exact etiological factors and molecular alteration in the esophageal carcinogenesis process to establish the strategies for prevention and early diagnosis of those with high risks.

Research frontiers

It has been well recognized that esophageal carcinogenesis is a progressive process involving multi-factors and multistage: tobacco, alcohol, fungal toxins, nutritional deficiencies, as well as infectious agents, are related to esophageal carcinogenesis. Among the infectious agents, human papillomavirus (HPV), a major cause of carcinoma of the cervix uteri throughout the world, is strongly implicated in the etiology of EC. HPV-16 and-18 are the most frequent genotypes, especially type-16 which is considered to be a risk factor for EC in the high prevalence area in Henan. On the other hand, oncoproteins of the high-risk HPV types can efficiently destroy the cell cycle and apoptotic pathways by binding to a number of host cell proteins, ultimately resulting in malignant transformation.

Innovations and breakthroughs

In this study, the authors found the HPV-16 DNA in 47% of esophageal squamous cell carcinomas (ESCC) and 29% of gastric cardiac adenocarcinomas (GCA) in concurrent cancers in the same patients (CC). Interestingly, HPV-16 DNA was detected in two cases of ESCC and GCA tissues simultaneously. These results suggested that HPV16 might participate in the carcinogenesis of the ESCC and GCA, and the two carcinomas might have similar risk of carcinogenesis. $P16^{INK4A}$ was highly expressed in both ESCC and GCA tissues. In the HPV-associated positive CC, higher $P16^{INK4A}$ expression was found in GCA than in ESCC (75% *vs* 25%, $P < 0.05$).

Applications

By knowing the prevalence and molecular alteration of high-risk HPV-associated EC, this study may contribute to the future strategies for prevention and early diagnosis of HPV-related malignancies, through the development of effective vaccines and biomarkers.

Terminology

HPVs are DNA viruses that infect basal skin and mucosal cells, and categorized according to their cervical oncogenicity-based risks.

Peer review

The manuscript is interesting, presenting data of HPV DNA detection in the concurrent esophageal squamous cell carcinoma and cardia adenocarcinoma and expression of p16 protein. Given the truth of these data, this would be a piece of evidence for involvement of HPV in esophageal carcinogenesis in man.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
San Francisco, CA, United States
The Medical Management of HIV/AIDS

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Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJG* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJG* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJG* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion; and (4) Maximization of the benefits of employees: It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality articles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

Aims and scope

The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

Columns

The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

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SPECIAL STATEMENT

All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

Biostatistical editing
Statisital review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc*. The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

Conflict-of-interest statement
In the interests of transparency and to help reviewers assess any potential bias, *WJG* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

Sample wording: [Name of individual] has received fees for serving as a speaker, a consultant and an advisory board member for [names of organizations], and has received research funding from [names of organization]. [Name of individual] is an employee of [name of organization]. [Name of individual] owns stocks and shares in [name of organization]. [Name of individual] owns patent [patent identification and brief description].

Statement of informed consent
Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics committee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. Authors should also draw attention to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964, as revised in 2004).

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When reporting the results from experiments, authors should follow the highest standards and the trial should conform to Good Clinical Practice (for example, US Food and Drug Administration Good Clinical Practice in FDA-Regulated Clinical Trials; UK Medicines Research Council Guidelines for Good Clinical Practice in Clinical Trials) and/or the World Medical Association Declaration of Helsinki. Generally, we suggest authors follow the lead investigator's national standard. If doubt exists whether the research was conducted in accordance with the above standards, the authors must explain the rationale for their approach and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study.

Before submitting, authors should make their study approved by the relevant research ethics committee or institutional review board. If human participants were involved, manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and appropriate informed consent of each. Any personal item or information will not be published without explicit consents from the involved patients. If experimental animals were used, the materials and methods (experimental procedures) section must clearly indicate that appropriate measures were taken to minimize pain or discomfort, and details of animal care should be provided.

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Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of Baishideng Publishing Group Co., Limited, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the ICMJE to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www.clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

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Title: Title should be less than 12 words.

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Authorship: Authorship credit should be in accordance with the standard proposed by ICMJE, based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. Authors should meet conditions 1, 2, and 3.

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Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

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Abstract

There are unstructured abstracts (no more than 256 words) and structured abstracts (no more than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no more than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections. AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/…"; MATERIALS AND METHODS (no more than 140 words); RESULTS (no more than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$; CONCLUSION (no more than 26 words).

Key words

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

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use

uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:...; B:...; C:...; D:...; E:...; F:...; G: …*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

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The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that...".

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

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Pleased provide PubMed citation numbers to the reference list, e.g. PMID and DOI, which can be found at http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed and http://www.crossref.org/SimpleTextQuery/, respectively. The numbers will be used in E-version of this journal.

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Authors: the name of the first author should be typed in boldfaced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

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Format

Journals

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

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

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 Pixudiarrhoea. *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

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Patent (list all authors)

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

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).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

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

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EDITORIAL

Reverse cholesterol transport revisited

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Abstract

Reverse cholesterol transport was originally described as the high-density lipoprotein-mediated cholesterol flux from the periphery *via* the hepatobiliary tract to the intestinal lumen, leading to fecal excretion. Since the introduction of reverse cholesterol transport in the 1970s, this pathway has been intensively investigated. In this topic highlight, the classical reverse cholesterol transport concepts are discussed and the subject reverse cholesterol transport is revisited.

Key words: Cholesterol; Reverse cholesterol transport; Transintestinal cholesterol efflux; Liver; Intestine; Lipoproteins

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FROM THE EDITOR

Cholesterol is of vital importance for all vertebrates. It is an essential structural component of cell membranes. In addition, cholesterol is needed for the manufacture of bile salts, steroid hormones and vitamin D. However, too much cholesterol is harmful. A high level of serum cholesterol is a risk factor for certain cardiovascular diseases. Fortunately, the body possess a transport route in order to dispose cholesterol: reverse cholesterol transport, which is an interesting target for drug development aiming at the treatment/prevention of atherosclerotic and associated diseases. Since the introduction of reverse cholesterol transport in the 1970s, this pathway has been intensively investigated.

The thorough research on reverse cholesterol transport has led to a great understanding about the way cholesterol is disposed. However, new insights in cholesterol excretion were rendered as well. Insights make the classical view on reverse cholesterol transport questionable. In this topic highlight, the classical reverse cholesterol transport concepts are discussed and the subject reverse cholesterol transport is revisited[1-7].

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TOPIC HIGHLIGHT

Astrid van der Velde, PhD, *Series Editor*

Reverse cholesterol transport: From classical view to new insights

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Abstract

Cholesterol is of vital importance for the human body. It is a constituent for most biological membranes, it is needed for the formation of bile salts, and it is the precursor for steroid hormones and vitamin D. However, the presence of excess cholesterol in cells, and in particular in macrophages in the arterial vessel wall, might be harmful. The accumulation of cholesterol in arteries can lead to atherosclerosis, and in turn, to other cardiovascular diseases. The route that is primarily thought to be responsible for the disposal of cholesterol is called reverse cholesterol transport (RCT). Therefore, RCT is seen as an interesting target for the development of drugs aimed at the prevention of atherosclerosis. Research on RCT has taken off in recent years. In this review, the classical concepts about RCT are discussed, together with new insights about this topic.

Key words: Cholesterol; Excretion; Transport; Intestine; Liver

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INTRODUCTION

In order to dispose of cholesterol, it is transported from the periphery to the liver and intestine, and is finally excreted *via* the feces. This pathway has been traditionally referred to as reverse cholesterol transport (RCT) or centripetal cholesterol flux. In this review, the term RCT is used. Research on RCT has taken off in recent years. This review starts with introduction of the term RCT in the early 1970s. This is followed by a discussion of research in the following years that led to the classical view on RCT: high-density lipoprotein (HDL)-mediated transport of cholesterol from the periphery to the liver, the subsequent uptake of HDL cholesterol by the liver, hepatobiliary cholesterol secretion, and finally, excretion *via* the feces (Figure 1). Both free cholesterol and the esterified form are involved in RCT. Cholesteryl esters are much less amphiphatic than free cholesterol and appear to be the preferred form for transport in the plasma and for storage. The transport proteins involved in RCT receive special attention in this review. Finally, new insights in cholesterol excretion are discussed, which make the current concept of RCT questionable.

THE BEGINNING

Glomset *et al*[1] introduced the term RCT in 1973, in a review that described the role of lecithin:cholesterol acyl

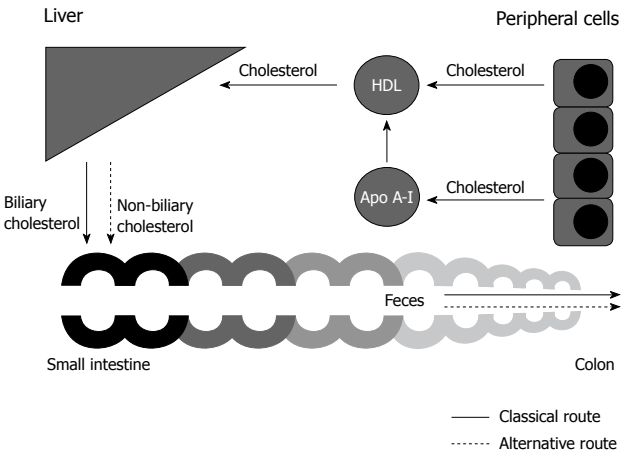

Figure 1 Cholesterol excretion routes. The filled arrows represent the classical route, and the dashed arrows represent the alternative route. In the classical view, apolipoprotein A-I (apo A-I) and high-density lipoprotein mediate cholesterol transport from the periphery towards the liver. Cholesterol is subsequently secreted into bile and is excreted *via* the feces. The alternative route describes the transport of non-biliary cholesterol towards the intestine for fecal excretion. How non-biliary cholesterol reaches the intestine is the subject of investigation.

transferase (LCAT) in metabolism. We nowadays know that LCAT is an enzyme that is synthesized in the liver and circulates in the blood plasma on the surface of lipoproteins, predominantly HDL. LCAT has the ability to catalyze the formation of cholesteryl esters on the surface of HDL by transferring fatty acids from phosphatidylcholine (PC, also known as lecithin) to the unesterified cholesterol[2]. It took several years after the discovery of LCAT before its physiological role was elucidated in more detail[3]. The *in vitro* testing of the possibility that LCAT plays a role in cholesterol transport was mainly hampered by the fact that most of the cholesterol of freshly prepared plasma lipoproteins is already esterified, that is, if the enzyme is able to alter the structure and physical properties of lipoproteins, most of the alterations have already occurred *in vivo*. The discovery of patients with familial LCAT deficiency[4-6] has provided a unique opportunity to circumvent these problems. By studying these patients, our knowledge about LCAT and cholesterol removal has increased.

CLASSICAL VIEW

Transport of cholesterol towards the liver

Although tissue culture studies had favored HDL[7,8] as the principal vehicle for cholesterol transport from the periphery to the liver, it took a decade before *in vivo* evidence was presented by Miller *et al*[9]. When cholesterol-loaded macrophages are incubated in medium that contains plasma, cholesterol moves from the cells to HDL and is subsequently esterified by LCAT[10]. The accumulation of cholesteryl esters in these particles increases their size and decreases their density; enrichment with apoprotein E also occurs, which decreases electrophoretic mobility[11,12]. Miller *et al*[9] have shown that similar changes were present in the circulating HDL of rabbits, when their peripheral tissues were loaded with cholesterol by intravenous injection of acetylated or native human low-density lipoproteins (LDLs).

Hepatic cholesterol uptake

Evidence for the existence of receptors that recognize HDL in plasma membranes of hepatocytes has been presented by several groups[13-15]. It appears that the rate of uptake of HDL cholesteryl esters by the liver in rats is several times greater than that of HDL apolipoprotein A-I (apo A-I), which suggests that cholesteryl esters dissociate from HDL particles at the surface of hepatocytes. This process might be facilitated by transient binding of HDL to the plasma membranes of such cells[16]. A similar process has been studied in cultures of human hepatoma cells[17]. The pathways for delivery of cholesteryl ester to the liver clearly cannot operate in patients with familial LCAT deficiency. Nevertheless, such patients do not accumulate large quantities of cholesterol in their plasma, as might be expected if the delivery of cholesteryl ester to the liver were hampered[18].

Hepatic processing of cholesterol

Once cholesterol has reached the liver, conversion into bile salts is the final destination for most of the cholesterol[19]. In contrast to cholesterol, bile salts are amphiphatic. Bile salts function as signaling molecules, and they act as physiological detergents: they emulsify droplets of dietary lipids in the intestine, which makes them available for absorption. Synthesis of bile salts involves the action of multiple different enzymes and can follow two major pathways, named the "classic" or neutral pathway and the alternative or acidic pathway. The classic pathway accounts for the majority of bile salt synthesis. In the enterohepatic circulation, intestinal bacteria modify bile acid structures, which yields secondary bile salts, for example, lithocholate and deoxycholate[20]. As about 95% of the bile salts reenter the enterohepatic circulation, the bile salt pool of the body consists of a mixture of primary and secondary bile salts. Only a small part of hepatic cholesterol is not converted to bile salts and is directly secreted into bile. The majority of this hepatobiliary secreted cholesterol will be re-absorbed in the small intestine[21].

Lipoproteins and lipid transfer

The first evidence that HDL is not a homogeneous set of molecules, but rather a mixture of heterogeneous subclasses, arose in 1979. Gebhardt *et al*[22] have described the existence of a pre-β migrating subclass of apo A-I-containing particles when studying human amniotic fluid. Similar particles have been demonstrated in peripheral lymph of dogs[23,24] and in human lymphedema fluid[25]. Castro *et al*[26] have found that, when radiolabeled cholesterol effluxes from cultured human fibroblasts into medium that contains human serum, almost all of it enters a minor component of HDL that is composed of very small apo A-I-containing particles. These particles differ from the majority of plasma HDL in having pre-β elec-

trophoretic mobility on agarose gel. It is rich in phospholipids, and contains little or no core lipid, and apo A-l as the only recognized apoprotein[26-28]. We know now that it is pre-β HDL cholesterol that is esterified by LCAT. After esterification, cholesteryl esters are sequestered into the core of the lipoprotein particle, eventually making spherical α-HDL. In this way, cholesterol is made ready for removal[2].

Some cholesteryl esters from HDL particles is transferred to LDL, very-low-density lipoproteins, or chylomicrons in exchange for triglycerides and phospholipids[29-35]. Triglyceride transfer is mediated by the cholesterol ester transfer protein (CETP) and phospholipid transfer is mediated by the phospholipid transfer protein (PLTP)[35,36]. Rodents do not express CETP, which might partly explain the high plasma HDL levels observed in these animals, in comparison to humans who express CETP. PLTP has not only been implicated in the transfer of phospholipids to HDL, but also in a process called HDL conversion[37]. In this process, PLTP mediates fusion of intermediate sized α-HDL particles to generate larger HDL particles with a concomitant release of lipid-poor apo A-I. These actions result in an enhanced capacity to take up cellular cholesterol.

Transport proteins

In the 1990s, several proteins and receptors involved in RCT were identified, which has given new insights in the mechanisms behind RCT.

ABCA1: Tangier disease was originally described and named on the basis of kindred living in Tangier Island in Chesapeake Bay, USA. Assmann *et al*[38] and Brook *et al*[39] have linked Tangier disease with abnormal HDL levels. The inheritance of the disease was already described in 1964[40]. However, it took until 1999 before it became clear that mutations in the ABC1 (nowadays in humans and rodents referred to as ABCA1) gene were responsible for the severe HDL deficiency in Tangier disease[41-43]. We now know that ABCA1 is involved in the first step of RCT. The mechanism by which ABCA1 mediates cholesterol efflux has been a matter of intense investigation. Two distinct mechanisms have been proposed to explain ABCA1-mediated cholesterol efflux from macrophages to apo A-I. These models are described below.

One model argues that apo A-I binds ABCA1 at the plasma membrane and is subsequently internalized and targeted to intracellular compartments, where lipidation of apo A-I occurs as part of a retroendocytosis pathway[44-46]. Hassan *et al*[47] have shown that two-thirds of apo A-I is bound to the plasma membrane and one-third is found in intracellular compartments. It appears that the C-terminal region of apo A-I is important in the ABCA1-mediated lipid efflux pathway. Apo A-I dissociated fourfold faster from the intracellular compartments than from the plasma membrane, which suggests an important contribution of ABCA1 in the endocytic pathway to apo A-I lipidation. In contrast, Faulkner *et al*[48] have shown, by studying ABCA1-mediated cholesterol efflux from macrophages, that the internalized apo A-I is re-secreted as a degraded protein. Furthermore, they have demonstrated that lipid-free apo-A-I-mediated cholesterol efflux from macrophages could be pharmacologically uncoupled from apo A-I internalization into cells, which raises doubts as to the significance of the endocytic pathway in efflux. In addition, confocal microscopy and efflux assays of apo A-I internalization and lipidation as a function of ABCA1 expression have indicated that apo A-I lipidation occurs at the cell surface, whereas ABCA1-dependent apo A-I internalization leads to its lysosomal targeting and degradation[49]. Data of Azuma *et al*[50] have suggested that the retroendocytosis pathway of ABCA1/apo A-I contributes to HDL formation when excess lipoprotein-derived cholesterol has accumulated in cells.

The other model argues that apo A-I forms complexes with phospholipids and cholesterol at the plasma membrane in a process that is promoted by ABCA1 activity. There is abundant evidence that ABCA1-mediated cholesterol efflux to apo A-I can occur at the plasma membrane[51-53]. It has been shown that optimal cholesterol efflux in macrophages requires binding of the C-terminal domain of apo A-I to a cell-surface-binding site, and the subsequent translocation of intracellular cholesterol to an efflux-competent pool[54]. By studying the binding of wild-type and mutant forms of human apo A-I to mouse J774 macrophages, it has been shown that ABCA1 activity creates two types of high affinity apo A-I binding sites at the cell surface. Only 10% of cell-surface-bound apo A-I interacts directly with ABCA1, whereas the rest is bound to lipid domains *via* the C-terminal domain. The low capacity site formed by direct apo A-I/ABCA1 interaction functions in a regulatory role, whereas the much higher capacity site generated by apo A-I/lipid interactions functions in lipidation[55]. Vedhachalam *et al*[53] have proposed an apo A-I/ABCA1 reaction scheme that involves three steps. First, there is binding of a small regulatory pool of apo A-I to ABCA1, thereby enhancing net phospholipid translocation to the plasma membrane exofacial leaflet; this leads to unequal lateral packing densities in the two leaflets of the phospholipid bilayer. Second, the resultant membrane strain is relieved by bending and by creation of exovesiculated lipid domains. The formation of highly curved membrane surface promotes high affinity binding of apo A-I to these domains. Third, this pool of bound apo A-I spontaneously solubilizes the exovesiculated domain to create discoidal nascent HDL particles. These particles contain 2-4 molecules of apo A-I and a complement of membrane phospholipid classes, together with some cholesterol. A key feature of this mechanism is that membrane bending induced by ABCA1 lipid translocase activity creates the conditions required for nascent HDL assembly by apo A-I. Overall, this mechanism is consistent with the known properties of ABCA1 and apo A-I and reconciles many of the apparently discrepant findings in the literature. Recently, it has been shown that sera with similar HDL cholesterol levels or apo A-I levels differ in

their ability to promote macrophage efflux due to the differences in the concentration of pre-β HDL[56].

ABCG1 and ABCG4: ABCA1 activity is needed for the predominant pathway for cholesterol efflux to apo A-I and the formation of pre-β HDL. However, it is doubtful whether ABCA1 operates alone in the formation of mature α-HDL. For example, HEK293 cells with ABCA1 overexpression, but without expression of HDL modifying factors like LCAT, PLTP, ABCG1, scavenger receptor class B type 1 (SR-BI), or apolipoprotein M, form pre-β HDL, but not α-HDL. In addition, it appears that the pre-β HDL that is formed is a poor substrate for subsequent lipidation by ABCA1, and presumably requires additional non-ABCA1-mediated lipidation for further maturation[57]. ABCG1 and ABCG4 or their heterodimers[58] might be good candidates for lipidation and maturation of HDL. Synergistic relationships between ABCA1 and ABCG1 and ABCG1/ABCG4 heterodimers have been demonstrated *in vitro*[59,60]. Wang *et al*[61] have shown that ABCG1 and ABCG4 mediate isotopic and net mass efflux of cellular cholesterol to HDL but not to lipid-poor apo A-I. In addition, mice with a targeted disruption of Abcg1 display impaired cholesterol efflux to mature HDL[62]. The exact mechanism behind ABCG1- and ABCG4-mediated cholesterol efflux is still a matter of debate.

SR-BI: SR-BI was identified as an HDL receptor (HDLR) in 1996. SR-BI is primarily expressed in liver and nonplacental steroidogenic tissues, and binds HDL with high affinity. The classic function of SR-BI is to mediate the selective uptake of HDL cholesterol by cells by a mechanism distinct from the classic LDL receptor (LDLR) pathway[63]. LDLR mediates endocytosis of the intact LDL particles *via* coated pits and vesicles, and their subsequent hydrolysis in lysosomes[64]. SR-BI mediates the selective uptake of HDL cholesterol by cells; primarily in the form of cholesteryl esters. This process involves the transfer of the cholesteryl esters from the hydrophobic core of the HDL particle to the cell, without transfer of the apolipoprotein at the surface of the particle. SR-BI-mediated selective lipid uptake appears to be a two-step process, in which high-affinity lipoprotein binding is followed by receptor-mediated transfer of lipids from the lipoprotein particle to the cell membrane. After lipid transfer, the lipid-depleted lipoprotein particle is released from the cells and re-enters the extracellular space[65]. SR-BI also mediates the bidirectional flux of unesterified cholesterol and phospholipids between HDL and cells[66].

Studies in genetically modified mice have revealed that SR-BI in the liver is particularly essential for RCT. In SR-BI$^{-/-}$ mice, plasma total cholesterol is elevated approximately twofold, and most of it circulates in abnormally large, heterogeneous, apolipoprotein-E-enriched HDL-like particles[67]. In addition, these mice exhibit impaired biliary cholesterol secretion, without concomitant changes in either biliary bile acid or phospholipid secretion[68]. Conversely, hepatic overexpression of SR-BI in mice results in the virtual disappearance of plasma HDL and a substantial increase in biliary cholesterol[69].

ABCG5 and ABCG8: At the beginning of the 21st century, two groups almost simultaneously identified mutations in genes ABCG5 and ABCG8, which underlie the disease sitosterolemia[70,71]. Sitosterolemia patients accumulate large amounts of plant sterols and exhibit complete abrogation of biliary plant sterol secretion. They also display increased cholesterol absorption and decreased biliary cholesterol secretion[72,73]. We now know that half-transporters ABCG5 and ABCG8 function as a heterodimer[70] in order to facilitate hepatobiliary cholesterol transport. Double knockout mice for Abcg5 and Abcg8 display extremely low biliary cholesterol concentrations in comparison to wild-type animals[74]. Overexpression of the human ABCG5 and ABCG8 in mice[75] and pharmacological induction of endogenous Abcg5 and Abcg8[76,77] both result in increased biliary cholesterol levels. Mice that lack either Abcg5[78], Abcg8[79], or both[74] do not show the same phenotype regarding physiology, which leaves room for discussion about the way these transporters function. Currently, two hypotheses exist. One argues that ABCG5/G8 act as a "flippase". In this case, the heterodimer shuttles cholesterol from the inner leaflet of the canalicular membrane through a chamber formed by the two half-transporters. This is followed by ATP binding and hydrolysis, which results in a conformational change of the complex. Cholesterol is thereby flipped into the outer membrane leaflet in a configuration that favors its release. Then, cholesterol is ready to be picked up by acceptors[80]. The other hypothesis argues that ABCG5/G8 act as a "liftase"[81]. In this case, the heterodimer promotes an activated state of cholesterol so that acceptors can easily pick up cholesterol. ABCG5/G8 could form a channel that binds cholesterol. Thereafter, the heterodimer might push cholesterol partly into the lumen when ATP is hydrolyzed (activation of cholesterol). Once cholesterol is activated, mixed micelles and PC vesicles are able to serve as an acceptor for cholesterol.

Summary

In summary, the classical view describes RCT as follows: the transport of cholesterol from the periphery to apo A-I and HDL by processes that are mediated by ABCA1, ABCG1 and/or ABCG4. The subsequent uptake of HDL cholesterol by the liver involves SR-BI. Thereafter, hepatobiliary cholesterol secretion is mediated by ABCG5/G8 and final excretion is *via* the feces.

NEW INSIGHTS

How solid is the classical definition of RCT? In recent years, new insights into RCT have been provided, which brings into question the current concept of RCT.

Classical route

Studies that try to pinpoint the rate-controlling step in RCT

often give rise to confusion. For example, Jolley *et al*[82] and Osono *et al*[83] has studied the importance of HDL in rate-control of RCT. They have used transgenic mice that expressed variable amounts of simian CETP in order to vary HDL levels. It appears that, in mice, neither the concentration of HDL cholesterol or apo A-I, nor the level of CETP activity dictate the magnitude of RCT[83]. Apo A-I$^{-/-}$ mice have very low HDL levels. When HDL is rate-controlling for RCT, then RCT should be hampered in apo A-I$^{-/-}$ mice. Nevertheless, Jolley *et al*[82] have demonstrated that the magnitude of the cholesterol flux from peripheral organs to the liver is virtually identical in mice that lacking apo A-I compared with control animals. They have suggested that the magnitude of RCT is probably ascribed to processes in peripheral organs. Although ABCA1 mediates cholesterol efflux from the periphery, it appears that Abca1$^{-/-}$ mice have unaltered hepatobiliary cholesterol secretion and fecal cholesterol excretion, while having virtually absent HDL levels[84]. Also, the proposed key player in the liver in HDL uptake, SR-BI, does not control RCT in mice[85]. Moreover, the exclusiveness of SR-BI as the sole high-affinity HDLR in the liver was compromised when Martinez *et al*[86] identified ecto-F1-ATPase as a high-affinity HDLR in hepatocytes. Furthermore, deficiency of Abcg5 and/or Abcg8 leads to mild[74,77] or no[78] decrease in fecal neutral sterol secretion in mice. This also questions the rate-controlling properties of these transporters on the magnitude of RCT. In addition, it has been shown that Abcg5/g8-independent, inducible routes exist that can significantly contribute to total hepatobiliary cholesterol output in mice[87]. Although most of the above-mentioned studies have been performed in mice and not in humans, it emphasizes the complexity of the mechanisms that underlie RCT.

Alternative route

In the classical concept of RCT, the liver plays a major role. When hepatobiliary cholesterol secretion is the primary route of cholesterol elimination, inhibition of ABCG5/G8, and hence, diminished hepatobiliary cholesterol secretion, should result in a drastic lowering of fecal neutral sterol excretion. Abcg5/Abcg8 double knockout mice do not show the expected low levels of fecal neutral sterol excretion[74]. In addition, Abcb4$^{-/-}$ mice, which also have almost no biliary cholesterol secretion, have the same fecal neutral sterol output as their wild-type littermates[88]. In addition, Kruit *et al*[88] have shown that, in these mice, intravenous radiolabeled cholesterol could be recovered in the feces. These findings indicate that hepatobiliary cholesterol secretion might not be the only route of cholesterol excretion. In mouse models with disturbed biliary secretion, there must be a direct transintestinal pathway for cholesterol excretion. In 2006, van der Velde *et al*[89] described significant direct intestinal cholesterol secretion in normal mice. This was later supported by van der Veen *et al*[90] who have measured cholesterol fluxes in mice. This route is nowadays referred to as transintestinal cholesterol efflux (TICE; see[91] for review) and continues to be elucidated[92-94]. Although quantitative cholesterol flux studies are warranted to quantify exactly the magnitude of TICE in humans, significant secretion of intestinal cholesterol in humans has been observed by Simmonds *et al*[21].

CONCLUSION

Although medical textbooks have taught us the classical view of RCT for several decades, it is desirable to open our eyes to the new insights into cholesterol excretion. Research on RCT has taken off in recent years. These new insights show us that the classical route for cholesterol excretion cannot be a strictly defined pathway. In addition, an alternative route for cholesterol disposal has come to light in the form of TICE (Figure 1). The new insights into RCT should make us question whether the classical view needs revision.

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TOPIC HIGHLIGHT

Astrid van der Velde, PhD, *Series Editor*

Scavenger receptor BI: A multi-purpose player in cholesterol and steroid metabolism

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Abstract

Scavenger receptor class B type I (SR-BI) is an important member of the scavenger receptor family of integral membrane glycoproteins. This review highlights studies in SR-BI knockout mice, which concern the role of SR-BI in cholesterol and steroid metabolism. SR-BI in hepatocytes is the sole molecule involved in selective uptake of cholesteryl esters from high-density lipoprotein (HDL). SR-BI plays a physiological role in binding and uptake of native apolipoprotein B (apoB)-containing lipoproteins by hepatocytes, which identifies SR-BI as a multi-purpose player in lipid uptake from the blood circulation into hepatocytes in mice. In adrenocortical cells, SR-BI mediates the selective uptake of HDL-cholesteryl esters, which is efficiently coupled to the synthesis of glucocorticoids (i.e. corticosterone). SR-BI knockout mice suffer from adrenal glucocorticoid insufficiency, which suggests that functional SR-BI protein is necessary for optimal adrenal steroidogenesis in mice. SR-BI in macrophages plays a dual role in cholesterol metabolism as it is able to take up cholesterol associated with HDL and apoB-containing lipoproteins and can possibly facilitate cholesterol efflux to HDL. Absence of SR-BI is associated with thrombocytopenia and altered thrombosis susceptibility, which suggests a novel role for SR-BI in regulating platelet number and function in mice. Transgenic expression of cholesteryl ester transfer protein in humanized SR-BI knockout mice normalizes hepatic delivery of HDL-cholesteryl esters. However, other pathologies associated with SR-BI deficiency, i.e. increased atherosclerosis susceptibility, adrenal glucocorticoid insufficiency, and impaired platelet function are not normalized, which suggests an important role for SR-BI in cholesterol and steroid metabolism in man. In conclusion, generation of SR-BI knockout mice has significantly contributed to our knowledge of the physiological role of SR-BI. Studies using these mice have identified SR-BI as a multi-purpose player in cholesterol and steroid metabolism because it has distinct roles in reverse cholesterol transport, adrenal steroidogenesis, and platelet function.

Key words: Scavenger receptor class B type I ; High-density lipoprotein; Cholesterol; Lipoprotein metabolism; Liver; Macrophages; Adrenal gland; Platelets; Steroidogenesis

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INTRODUCTION

The scavenger receptor (SR) superfamily consists of integral membrane glycoproteins that are involved in recognition of polyanionic structures of either endogenous [e.g. oxidized low-density lipoprotein (LDL)] or exogenous [e.g. bacterial lipopolysaccharide (LPS)] origin. The SR family is structurally diverse and can be classified into eight different classes (A-H) based on the multi-domain structure of the individual members (reviewed by van Berkel *et al*[1]). The first SR to be cloned was the class A scavenger receptor SR-AI, which was originally identified as a receptor that recognizes acetylated LDL (acLDL). However, due to the limited relevance of acLDL in atherosclerosis, at that time it was primarily considered to be a receptor for negatively charged macromolecules[2]. Macrophage-expressed SR-As can bind many polyanionic molecules such as lipoteichoic acid from Gram-positive bacteria, and lipid IVA, a precursor of lipid A, from LPS of Gram-negative bacteria, as well as mediate uptake of bacteria by phagocytosis. In line with their ability to bind bacteria, class A scavenger receptors play prominent roles in the host response to infection[3,4]. In parallel with SR-As, SR class B type I (SR-BI), also known as CD36 and lysosomal integral membrane protein-Ⅱ analog-1 (CLA-1) in humans, has also been demonstrated to increase the uptake of both Gram-negative and Gram-positive bacteria *in vitro*[5,6].

As its name indicates, SR-BI belongs to the class B subfamily of SRs that also includes its splice variant SR-BII and CD36 (previously known as the OKM5 antigen, platelet glycoprotein Ⅳ, or GP88). The three class B SR proteins show a highly similar structure, which consists of a heavily N-linked glycosylated and fatty acylated protein backbone, which contains a large extracellular loop, two transmembrane domains, and short intracellular N-terminal and C-terminal domains (Figure 1). CD36 and SR-BI represent two distinct proteins that are derived from two different genes located on chromosome 5 in mice and 7 and 12 in humans. SR-BII, however, constitutes an isoform of the *SR-BI* gene, which represents around 40% of total SR-BI/BII mRNA[7]. The SR-BII protein differs from SR-BI only in the C-terminal cytoplasmic tail and contributes to only 12% of the immunodetectable SR-BI/BII protein in mouse liver[7].

Importantly, although SR-BI also modulates susceptibility to sepsis[8,9], it is predominantly known for its functions in lipoprotein metabolism. Since the generation of SR-BI knockout mice that lack a functional SR-BI protein by the group of Monty Krieger in 1997[10], it has become clear that SR-BI is a multi-purpose player in cholesterol and steroid metabolism. In this topic highlight, we review the data obtained from this widely used mouse model regarding the *in vivo* role of SR-BI in cholesterol and steroid metabolism within a wide variety of cells types in mice, including liver parenchymal cells (hepatocytes), adrenocortical cells, macrophages, and platelets. In addition, we discuss recent interesting findings from humanized SR-BI knockout mice that provide the first insight into an important contribution of SR-BI to steroid metabolism and (patho)physiology in humans.

LIVER PARENCHYMAL CELLS

Initial *in vitro* cloning and purification studies performed by the group of Krieger have shown that SR-BI, similar to CD36, displays a high affinity for acLDL, modified proteins (i.e. maleylated bovine serum albumin), and anionic lipids[11,12]. Not long after, it became clear that SR-BI, in addition to its ability to bind (modified) LDL and anionic lipids, can bind high-density lipoprotein (HDL) with a high affinity and saturability[13]. This provided the first evidence that SR-BI could be considered a functional HDL receptor. Immunoblotting on membranes of different murine tissues has revealed that SR-BI protein is highly expressed in liver[13]. The liver is the primary organ that is involved in removal of cholesterol from the body, therefore, much attention has been drawn to a possible role for SR-BI in hepatic lipid transport.

The liver consists of a wide variety of cells including parenchymal cells (hepatocytes), endothelial cells, and Kupffer cells, which represent the largest majority of all tissue macrophages found within the body. Expression profiling has indicated that SR-BI mRNA and protein are particularly high in parenchymal cells, the primary metabolic cell type of the liver[14,15]. However, SR-BI is also detected in Kupffer cells, albeit at a lower expression level[14,16]. In accordance with a role for SR-BI in hepatocyte HDL-cholesterol clearance, liver parenchymal cells are responsible for 88% of the hepatic uptake of cholesteryl esters from HDL in wild-type mice, when taking into account the contribution of these cells to liver mass[17]. Importantly, we have been able to show that SR-BI is responsible for the majority of the uptake of HDL-cholesteryl esters into the liver. Functional SR-BI deficiency in SR-BI knockout mice was associated with an 87% and 52% decrease in uptake of HDL-cholesteryl esters in liver parenchymal and Kupffer cells, respectively, compared with wild-type littermate controls[17]. *In vitro* association studies showed uptake of HDL-cholesteryl esters without whole particle internalization (selective uptake) in isolated liver parenchymal cells of wild-type mice, which was completely lost in those of SR-BI knockout mice. This suggests that, at least in mice, SR-BI is the sole molecule that is involved in the selective uptake of HDL-associated cholesteryl esters in hepatocytes[17]. In parallel with the major role of the liver in the clearance of HDL-associated cholesterol from the blood circulation, SR-BI knockout mice exhibit a marked increase in their plasma HDL-cholesterol levels. Already on a regular chow diet, in these mice an accumulation of larger, but not more, cholesteryl-ester-rich HDL particles can be observed[10], probably as a result of the diminished selective uptake of HDL-cholesteryl esters by SR-BI in the liver. In addition, SR-BI attenuated mice, with an SR-BI promoter mutation that resulted in 53% decreased expression of the receptor in the liver, displayed a lower hepatic selective HDL-cho-

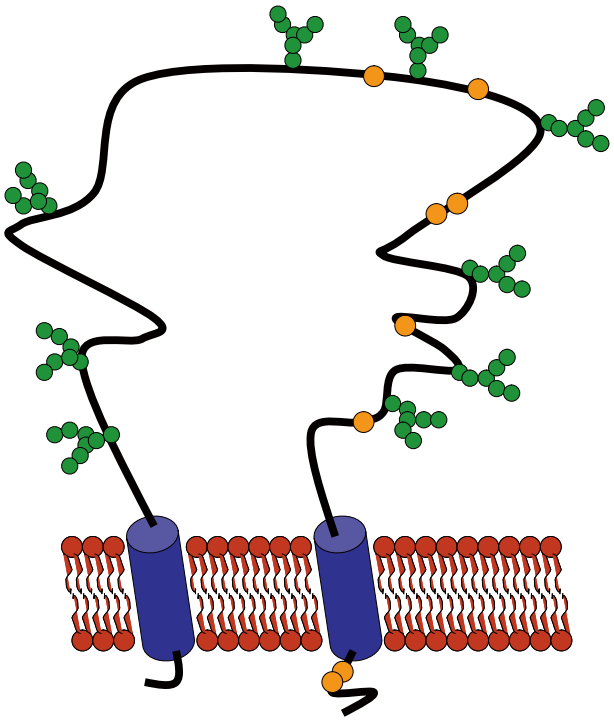

Figure 1 Schematic representation of the scavenger receptor class B type I protein. Structural elements include a heavily N-glycosylated and fatty-acylated protein backbone that contains a large extracellular loop, two transmembrane domains, and short intracellular N-terminal and C-terminal domains.

lesteryl ester uptake and a higher plasma HDL-cholesterol level[18]. Furthermore, transgenic or adenoviral overexpression of SR-BI in hepatocytes is associated with virtual disappearance of HDL from the plasma compartment[19-22]. These findings convincingly show that SR-BI in mice is a functional HDL receptor in liver parenchymal cells.

SR-BI knockout mice also display a significant increase in the level of cholesterol associated with the plasma non-HDL fraction[10,23,24]. In parallel, transgenic mice that have liver-specific overexpression of SR-BI show a decrease in LDL- and very low density lipoprotein (VLDL)-cholesterol levels[20]. These observations suggest that SR-BI can also contribute to the clearance of apoB-containing lipoproteins *in vivo*. To verify this hypothesis, several groups have studied uptake of apoB100-containing LDL and VLDL lipoproteins, as well as apoB48-containing chylomicrons in hepatocytes from SR-BI knockout mice and their wild-type littermate controls. Rhainds *et al*[25] and Bourret *et al*[26] have detected a dose-dependent decrease in the selective uptake of cholesteryl esters associated with human LDL by liver parenchymal cells isolated from heterozygous (-45%) and homozygous (-87%) SR-BI knockout mice[25,26]. In parallel, the uptake of triglyceride-rich, chylomicron- remnant-like emulsion particles by liver parenchymal cells of homozygous SR-BI knockout mice is markedly lower compared to that of wild-type controls[27]. Furthermore, the association with and apparent uptake of β-VLDL is 1.6-2.2-fold decreased in hepatocytes isolated from SR-BI knockout mice *in vitro*[24], whereas the serum decay and hepatic uptake of β-VLDL is significantly diminished in response to SR-BI deficiency *in vivo*[24]. Moreover, SR-BI knockout mice show a higher postprandial triglyceride response to an intragastric fat load[27]. SR-BI deficiency, however, does not alter the ability of oxidized LDL to associate with liver parenchymal cells *in vitro*[26]. It thus seems that SR-BI also plays a physiological role in the binding and uptake of native, but not modified, apoB-containing lipoproteins by liver parenchymal cells *in vivo*. Combined with the fact that SR-BI is a functional HDL receptor *in vivo*, SR-BI can be considered a multi-purpose player in lipid uptake from the blood circulation into hepatocytes in mice.

ADRENOCORTICAL CELLS

Although SR-BI plays a widely recognized role in hepatic clearance of lipids, the highest protein expression of SR-BI is actually found in the adrenal glands[13]. More specifically, adrenocortical cells (i.e. Y1-BS1 murine adrenal cells) contain relatively high levels of SR-BI protein that are predominantly localized in cholesterol-rich caveolin-1-containing domains within the plasma membrane[28]. Cells within the adrenal cortex are involved in the synthesis of cholesterol-derived steroid hormones, including mineralocorticoids (i.e. aldosterone) and glucocorticoids (i.e. cortisol in humans and corticosterone in rodents). In the original 1997 study on the effect of the targeted mutation in the SR-BI gene on cholesterol metabolism *in vivo*, it was described that the adrenal cholesterol content was dose-dependently decreased in heterozygous (-42%) and homozygous (-72%) SR-BI knockout mice[10]. In accordance, the uptake of cholesteryl esters from HDL by the adrenals of SR-BI knockout mice is reduced[17]. Strikingly, however, in 2008, we and others were able to show that the impaired uptake of HDL-cholesteryl esters by the adrenal glands translates into functional changes in the adrenal steroidogenesis rate in SR-BI knockout mice. Adrenal SR-BI deficiency is associated with increased adrenal weight as a result of long-term overstimulation of adrenocortical cell proliferation by the pituitary-derived adrenocorticotropic hormone (ACTH)[10,29]. Under basal non-stressed conditions, plasma glucocorticoid levels (i.e. corticosterone) in SR-BI knockout mice are maintained within the normal range, probably as a result of the high level of circulating ACTH, a potent activator of adrenal steroidogenesis[8,29]. In contrast, SR-BI knockout mice are unable to increase plasma corticosterone levels upon a variety of stress triggers that activate the hypothalamus-pituitary-adrenal axis, which results in increased secretion of glucocorticoids by adrenocortical cells in the zona fasciculata. We have shown that the fasting-induced adrenal glucocorticoid response is significantly diminished in SR-BI knockout mice, resulting in a approximately 50% lower fasting plasma glucocorticoid level[29,30]. The adrenal cortex is virtually depleted of neutral lipids (i.e. cholesteryl esters) in SR-BI knockout mice under fasting stress conditions, which is associated with a visual change in the appearance of the adrenal glands (red/brownish color instead of

white)[10,29]. In parallel with fasting-induced glucocorticoid insufficiency, adrenals of SR-BI knockout mice are also unable to respond to a potent inflammatory stress trigger. Upon LPS exposure, wild-type mice show a significant increase in their plasma corticosterone levels. Plasma corticosterone levels, however, are not induced in SR-BI knockout mice challenged with LPS[8,30]. Glucocorticoids are important signaling molecules that through the action of their cognate nuclear receptor mediate downstream effects on energy homeostasis and the control of immune responses. In accordance, SR-BI knockout mice exhibit fasting hypoglycemia, which is paralleled by reduced expression of hepatic glucocorticoid targets involved in fatty acid utilization[29]. Furthermore, the LPS-induced cytokine (i.e. tumor necrosis factor-α and interleukin-6) response is enhanced, probably due to an impaired suppressive action of glucocorticoids on pro-inflammatory gene expression in macrophages and other immune cells, which leads to an overall higher LPS-induced mortality rate in SR-BI knockout mice[8]. In line with a prominent role for SR-BI in the generation of the substrate used for adrenal steroidogenesis under stress conditions, SR-BI expression in adrenocortical cells is tightly controlled by the steroidogenic activator ACTH *in vitro* and *in vivo*[31,32]. These findings suggest that functional SR-BI protein is necessary for optimal adrenal steroidogenesis in mice.

MACROPHAGES

Macrophages cannot limit the uptake of excess cholesterol and thus rely on active cholesterol efflux processes to maintain their intracellular cholesterol balance. As a result, disruption of the function of proteins crucially involved in macrophage cholesterol efflux is associated with the formation of so-called foam cells, which are large lipid-filled macrophages. The cholesterol transport protein ATP-binding cassette transporter A1 (ABCA1) has been shown to be a major player in macrophage cholesterol efflux. Ablation of ABCA1 function is associated with almost complete shutdown of cholesterol efflux from macrophages to apolipoprotein AI (apoAI), and enhanced foam cell formation *in vitro* and *in vivo*[33-35]. Lipidation of apoAI is the primary step in the formation of HDL, which executes the transport of cholesterol from peripheral cells (i.e. macrophages) back to the liver for subsequent excretion into the bile, a process also known as reverse cholesterol transport (reviewed by Van Eck *et al*[36]). SR-BI is a major player in the final step of reverse cholesterol transport because it, as discussed previously, mediates the selective uptake of HDL-cholesteryl esters into the liver.

Initial overexpression studies by Ji *et al*[37] have shown a clear correlation between the level of cellular cholesterol efflux to mature HDL and the expression levels of SR-BI protein *in vitro*, which suggests that SR-BI also mediates the primary step in reverse cholesterol transport *in vivo*. Follow-up studies have indicated that SR-BI does indeed mediate cholesterol efflux to HDL in cells that have been labeled with unesterified cholesterol[38]. Strikingly, however, SR-BI has been shown not to influence the efflux of cholesterol from macrophages that are loaded with cholesterol packaged in acLDL[38]. Furthermore, it actually decreases the efflux of cholesterol to apoAI *via* ABCA1 in RAW macrophages, which could be attributed the ability of SR-BI to re-uptake cholesterol after ABCA1-mediated efflux[38]. These studies were the first to show that SR-BI and ABCA1 have distinct and competing roles in mediating cholesterol flux between (pre-β) HDL and macrophages. In line with a role for SR-BI in cholesterol uptake, the association of β-VLDL and HDL in peritoneal macrophages is reduced in response to SR-BI deficiency[39]. Studies *in vitro*, however, have either shown that SR-BI does modulate cholesterol efflux[37] or have argued against a role for SR-BI in efflux in general[40-42]. To date, the quantitative role for SR-BI in macrophage cholesterol efflux has therefore been under discussion and this has contributed to the mystification of SR-BI as a player in cholesterol efflux from macrophages.

Thioglycolate-elicited peritoneal macrophages from SR-BI knockout mice loaded with unesterified cholesterol *in vivo* and subsequently subjected to cholesterol efflux *in vitro* displayed a 20% reduced efflux to mature HDL as compared to wild-type macrophages[39]. To study the quantitative role of different transport proteins in cholesterol efflux in mice, the group of Dan Rader has developed an experimental *in vivo* reverse cholesterol transport model. *In vitro* cholesterol-labeled bone marrow-derived or peritoneal macrophages from specific knockout mice are injected into recipient mice and subsequently the appearance of cholesterol in the plasma compartment, uptake by the liver, and the rate of sterol excretion into the feces are monitored. Using this experimental model, no effect of macrophage SR-BI deficiency on macrophage cholesterol efflux/reverse cholesterol transport was detected, because the cholesterol distribution in plasma as well as fecal tracer levels were no different upon injection of peritoneal or bone-marrow-derived macrophages from SR-BI knockout mice and their wild-type littermates into C57BL/6 recipient mice[43]. However, macrophages were cultured *in vitro* for at least 24 h before injection into recipient mice. *In-vitro*-cultured macrophages might display an altered expression of cholesterol transport proteins as compared to macrophages *in vivo*. In this respect, the absence of an effect of macrophage SR-BI deficiency in the described reverse cholesterol transport model might not necessarily imply that SR-BI does not affect the cholesterol efflux potential of macrophages *in vivo*.

In our opinion, too little knowledge is currently present about which type of macrophage and cholesterol acceptor combination *in vitro* best represents the *in vivo* situation. We anticipate that large-scale genomic and proteomic analysis on freshly isolated (tissue) macrophage foam cells, as well as peritoneal and bone-marrow-derived macrophages cultured under different loading conditions (i.e. unesterified cholesterol, acLDL, oxidized LDL) is needed to provide clues as to which setting might actually

be useful to study functional consequence of specified gene targets on cholesterol efflux *in vivo*. Although we appreciate an important role for SR-BI in cholesterol efflux, we encourage novel research into this interesting topic, which will unravel not only the contribution of SR-BI, but also clarify the interaction between the different cholesterol efflux pathways.

PLATELETS

Early evidence in several disease states in humans (e.g. familial hyperlipidemia) has suggested an interaction between cholesterol-containing lipoproteins and platelets[44,45]. As a consequence, the modulation of platelet functions by lipoproteins has been investigated intensively both in humans and animal models. Due to the large fluctuation in their plasma concentrations, the data on the influence of triglyceride-rich lipoproteins, such as chylomicrons and VLDL, are limited. In contrast, numerous studies have demonstrated a distinctive interaction of platelets with LDL and HDL (reviewed by Korporaal *et al*[46]).

Platelets are able bind to rapidly and reversibly to HDL in an activation-state- and temperature-independent manner with a Kd of 11-60 nmol/L[47,48]. The number of HDL binding sites expressed on the platelet surface ranges from 1200 to 3200 copies[47,48]. Koller *et al*[48,49] have observed that LDL interferes with the binding of HDL to platelets as a result of overlapping affinities for different receptors. In 1986, the same group described CD41 and CD61, the two constituents of integrin αⅡbβ3, as binding proteins for HDL on the platelet surface. An antibody directed against the integrin β3-subunit blocked the binding of HDL to integrin αⅡbβ3 on the platelet surface, thereby identifying αⅡbβ3 as the platelet receptor for HDL[49]. In contrast with these observations, others have reported that αⅡbβ3 is not involved in binding of HDL to platelets, because: (1) antibodies directed against integrin αⅡbβ3 have no effect; (2) HDL does not alter agonist-induced fibrinogen binding or platelet aggregation[50,51]; (3) HDL-induced platelet signaling is similar in control platelets and platelets from thrombasthenic patients with abnormal levels of αⅡbβ3 and fibrinogen[52]; (4) treatment of platelets with EDTA, which causes dissociation of the integrin complex and fully inhibits fibrinogen binding and platelet aggregation[53], does not inhibit the interaction between HDL and platelets[54]; and (5) αⅡbβ3 ligands like fibrinogen, fibronectin, vitronectin, and von Willebrand factor do not affect HDL binding[52]. It therefore remains unclear which receptor is the actual binding site for HDL in platelets.

In 2003, Imachi *et al*[55] identified SR-BI as being expressed on human platelets, which opened up the possibility that, as in the liver, adrenals, and macrophages, SR-BI acts as a functional HDL receptor in platelets. *In vitro* association studies by Valiyaveettil *et al*[56] have proved that oxidized HDL (oxHDL) binds to isolated platelets in an SR-BI-dependent manner. Binding of oxHDL could be diminished by pre-incubation of platelets with an SR-BI blocking antibody, whereas native HDL decreased oxHDL binding to human platelets[56]. In parallel, the oxHDL-induced repression of platelet aggregation *in vitro* was almost fully blocked by inhibiting SR-BI binding, which suggests that a direct interaction of HDL with SR-BI is necessary to alter normal platelet function[56]. To validate the functional role of SR-BI in platelets *in vivo*, the group of Monty Krieger has evaluated platelet function in SR-BI knockout mice and their littermate controls. SR-BI knockout mice suffer from thrombocytopenia, because their blood platelet counts are markedly reduced as a result of enhanced clearance of platelets by the reticuloendothelial system[57]. Probably as a compensatory response, splenic megakaryocyte (platelet precursor) counts are increased in SR-BI knockout mice[57]. Elegant crossover platelet infusion studies have shown that the increased turnover of platelets is not primarily due to genotype-induced changes in the platelets themselves, but rather is secondary to the high level of HDL-associated unesterified cholesterol that circulates in the plasma of SR-BI knockout mice. As unesterified cholesterol rapidly exchanges between the plasma compartment and blood cells, including platelets, SR-BI knockout platelets contain relative high cellular levels of cholesterol, which is associated with a functional impairment of the aggregation in response to ADP[57]. In parallel, we have shown that SR-BI deficiency and the dyslipidemia associated with it lead to thrombocytopenia and impaired platelet reactivity *ex vivo* as a result of the increased platelet cholesterol content[58]. In addition, SR-BI deficiency in mice is associated with enhanced thrombosis susceptibility[58]. However, platelet-specific deficiency of SR-BI is associated with resistance to hyper-reactivity induced by increased platelet cholesterol content[59], which suggests that SR-BI contributes to platelet function *in vivo*. In accordance, platelet-specific SR-BI modulates thrombosis susceptibility in SR-BI knockout mice[59]. These findings highlight an interesting novel role for SR-BI in regulating platelet number and function and thrombosis susceptibility in mice.

POTENTIAL FOR SR-BI IN CHOLESTEROL AND STEROID METABOLISM IN HUMANS: INSIGHTS FROM HUMANIZED SR-BI KNOCKOUT MICE

From the combined findings in SR-BI knockout mice it can be concluded that SR-BI is a multi-purpose player in cholesterol and steroid metabolism in mice. Several clinical studies have detected significant associations between several polymorphisms at the SR-BI locus and changes in plasma lipid levels and lipoprotein particle sizes[60-62]. Human subjects, in contrast to rodents, express cholesteryl ester transfer protein (CETP) that is able to transfer cholesteryl esters from HDL to the apoB-containing lipoproteins VLDL and LDL, in exchange for triglycerides. As a result, human subjects carry most of their cholesterol in the LDL fraction, whereas mice predominantly transport

their plasma lipids in HDL. The CETP→LDL→LDL receptor route provides an alternative for HDL-cholesteryl ester delivery from the blood circulation to the liver, which might be associated with a relatively limited uptake of HDL-cholesteryl esters *via* SR-BI into the liver in humans compared with mice. To date, no functional mutations in the *SR-BI* gene have been identified, therefore, the relative contribution of SR-BI to lipoprotein metabolism in humans is therefore still unclear.

To gain insight in the importance of SR-BI in the human situation, studies have been performed in humanized SR-BI knockout mice that express the *CETP* gene under the control of its natural regulatory elements. In these SR-BI knockout/CETP transgenic mice, similar to the human situation, relatively high mRNA expression levels of CETP can be detected in adipose tissue and macrophage-rich tissues such as the liver and spleen[30]. In accordance with the assumption that the CETP→LDL →LDL receptor route can provide an alternative for the delivery of HDL-associated cholesteryl esters to the liver in humans, transgenic expression of CETP is able to normalize almost fully the serum decay and hepatic uptake of HDL-cholesteryl esters in SR-BI knockout mice[63,64]. As a result, plasma total cholesterol levels are significantly lower in SR-BI knockout/CETP transgenic mice as compared to SR-BI knockout mice[30,63,64]. Importantly, plasma lipid levels as well as lipoprotein particle sizes are not fully restored. Plasma unesterified cholesterol levels, the unesterified cholesterol to total cholesterol ratio, and HDL particle size are only mildly decreased by CETP expression[63]. In parallel with previous findings of Dole *et al*[57], the increased unesterified cholesterol levels in SR-BI knockout mice with or without CETP expression are associated with a lower platelet count and reduced platelet aggregation[63]. CETP expression increases, but does not normalize, adrenal uptake of HDL-associated cholesteryl esters in SR-BI knockout mice. Strikingly, the stress-induced adrenal glucocorticoid insufficiency, however, is equally severe in SR-BI knockout mice with or without CETP expression[30]. Furthermore, the higher tissue oxidative status previously detected in SR-BI knockout mice[65] does not return to basal levels in mice that express the CETP transgene[63]. Changes in plasma lipid levels (i.e. an increase in apoB-containing lipoproteins or a decrease in HDL)[66], an increased adrenal steroidogenesis rate[67] as well as modified platelet function[68] underlie the process of atherothrombosis, the primary cause of cardiovascular disease mortality and morbidity. SR-BI deficiency in mice is associated with enhanced susceptibility to atherosclerotic lesion development[23]. This finding indicates that high HDL-cholesterol levels *per se* do not protect against atherosclerosis and underlines that other parameters regarding HDL function should be established and evaluated in the clinical setting. Expression of CETP also does not provide protection against atherosclerosis in SR-BI knockout mice[63]. Overall, it thus seems that, although CETP activity can restore the transport of HDL-cholesteryl esters to the liver in SR-BI knockout mice, it cannot normalize many other processes associated with disruption of the *SR-BI* gene in mice.

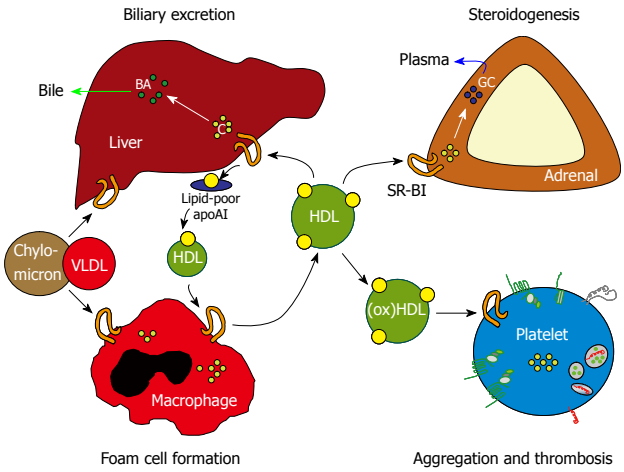

Figure 2 Overview of the diverse role of scavenger receptor class B type I in cholesterol and steroid metabolism in mice. (1) In liver, scavenger receptor class B type I (SR-BI) mediates the selective uptake of cholesterol (C; yellow) from high-density lipoprotein (HDL) that is subsequently converted to bile acids (BA; green) for biliary excretion; (2) HDL-associated cholesteryl esters are taken up *via* SR-BI in adrenocortical cells, which is efficiently coupled to the synthesis and subsequent secretion of glucocorticoids (GC; blue) into plasma; (3) SR-BI binds apolipoprotein (apo)B-containing lipoproteins, i.e. chylomicrons and very low density lipoprotein (VLDL), in liver and macrophages, which contributes to macrophage foam cell formation; (4) SR-BI is able to efflux cholesterol from macrophages to small HDL particles, which inhibits foam cell formation; and (5) HDL can interact with platelets, in part *via* the direct action of SR-BI, to modulate platelet aggregation and susceptibility to thrombosis.

CONCLUSION

The initial generation of the SR-BI knockout mice by the group of Monty Krieger has significantly contributed to our knowledge of the physiological role of SR-BI. Studies using these mice have identified SR-BI as a multi-purpose player in cholesterol and steroid metabolism, because it has distinct roles in reverse cholesterol transport, adrenal steroidogenesis, and platelet function (summarized in Figure 2). Recent studies have suggested a potential role for HDL and SR-BI in the control of endothelial cell[69-71] and stem and progenitor cell[72,73] physiology, and we are confident that upcoming research using SR-BI knockout mice will also reveal the importance of SR-BI in these additional cell systems.

Based upon the findings from humanized CETP transgenic SR-BI knockout mice, we anticipate that functional SR-BI mutations in humans who do naturally express CETP will be associated with various diseases (i.e. adrenal glucocorticoid insufficiency, impaired platelet function, and enhanced atherogenesis) similar to those observed in SR-BI knockout mice. We hope that functional mutations in the *SR-BI* gene will be identified in the near future as a result of large-scale DNA screening studies in human subjects that have relatively high plasma HDL-cholesterol levels. If changes in SR-BI function in humans are indeed associated with an increased susceptibility for atherosclerotic

rosis, this will further establish the importance of SR-BI as a therapeutic target for increasing HDL-mediated reverse cholesterol and lowering clinical atherosclerosis and the associated cardiovascular disease risk.

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TOPIC HIGHLIGHT

Astrid van der Velde, PhD, *Series Editor*

Ecto-F1-ATPase: A moonlighting protein complex and an unexpected apoA-I receptor

Pierre Vantourout, Claudia Radojkovic, Laeticia Lichtenstein, Véronique Pons, Eric Champagne, Laurent O Martinez

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Abstract

Mitochondrial ATP synthase has been recently detected at the surface of different cell types, where it is a high affinity receptor for apoA-I, the major protein component in high density lipoproteins (HDL). Cell surface ATP synthase (namely ecto-F1-ATPase) expression is related to different biological effects, such as regulation of HDL uptake by hepatocytes, endothelial cell proliferation or antitumor activity of Vγ9/Vδ2 T lymphocytes. This paper reviews the recently discovered functions and regulations of ecto-F1-ATPase. Particularly, the role of the F1-ATPase pathway(s) in HDL-cholesterol uptake and apoA-I-mediated endothelial protection suggests its potential importance in reverse cholesterol transport and its regulation might represent a potential therapeutic target for HDL-related therapy for cardiovascular diseases. Therefore, it is timely for us to better understand how this ecto-enzyme and downstream pathways are regulated and to develop pharmacologic interventions.

Key words: F_1F_o ATP synthase; High density lipoproteins receptor; Apolipoprotein A-I; Purinergic receptor P2Y13; Adenylate kinase; Nucleotide; Endothelium; Antitumor immunity

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INTRODUCTION

F_1F_o ATP synthase, the terminal enzyme of the oxidative phosphorylation pathway, is a complex molecular motor responsible for the large majority of ATP synthesis in all living beings, except in archaea which use a related enzyme to produce ATP, the A_1A_o ATP synthase[1]. It is

located in the inner membrane of mitochondria, in the thylakoid membrane of chloroplasts in plants, and in the plasma membrane (PM) of certain bacteria. ATP synthase is highly concentrated in inner membrane cristae, where it is part of the so-called "ATP synthasome", in association with an inorganic phosphate (Pi) carrier as well as the adenine nucleotide translocase (ANT) which exchanges ADP and ATP[2]. Experimental evidence also suggests that ATP synthase is organized in dimers[3], or even oligomers[4]. This supramolecular organization is involved in the generation of inner membrane cristae and depends on the e and g subunits[5].

Despite a few differences in subunit composition, the general structure of ATP synthase is highly conserved throughout evolution. In eukaryotic cells, it is composed of 16 unique subunits (α, β and c being present in multiple copies in the whole complex). It comprises the activity-bearing subunits (three αβ heterodimers), a rotor (subunits γ, δ, ε and the c ring) and a stator (subunits a, e, f, g, A6L, b, F6, d and OSCP) which holds the αβ heterodimers. It is organized into two major domains: a soluble F_1 domain (α, β, γ, δ, ε) and a membrane-associated F_o domain (Figure 1). The F_1 domain bears the catalytic activity while the F_o domain is a proton channel. The biogenesis of ATP synthase follows a complex and timed mechanism[6] which involves at least five assembly factors: Atp10p, Atp11p, Atp12p, Atp22p and Fmc1p[7-10]. So far only the human orthologs of Atp11p and Atp12p have been identified[11].

The F_o domain uses the proton gradient created by the four other complexes of the respiratory chain to drive the rotation of the central stalk, which will alternatively change the conformation of the three αβ heterodimers, leading to the synthesis of ATP from ADP and Pi. The rotation of ATP synthase was demonstrated by a remarkable experiment in which the αβ heterodimers were immobilized on a solid support while the γ chain was coupled to a fluorochrome-labeled actin filament, and visualized under a microscope after addition of ATP[12]. ATP synthesis and hydrolysis involve a binding-change mechanism[13,14] in which catalytic sites undergo conformational changes driven by the interactions with the γ chain. In the case of the eukaryotic ATP synthase, the translocation of 10 protons through the c ring will lead to a full turn, and the synthesis of 3 ATP molecules. However, the exact mechanism of catalysis is still debated, as experimental data support both a bi-site mechanism, in which one of the catalytic sites is empty at any given moment[15], or a tri-site mechanism where all sites are filled with nucleotides[16]. The F_1 domain also bears three non-catalytic sites which have been proposed to enhance ATP synthesis/hydrolysis upon binding of nucleotides[17].

In the absence of a proton gradient, or if the F_1 domain is isolated from the F_o domain, the enzyme behaves as an ATP hydrolase. This potential energy loss is tightly regulated by the inhibitor factor 1 (IF_1) protein when the proton gradient collapses, for instance in the case of hypoxia as the respiratory chain stops functioning. Under normal conditions, IF_1 exists as a homotetramer which turns into a homodimer when the pH in the matrix compartment decreases[18]. This allows it to tightly bind ATP synthase by interacting with both the αβ dimers and the central stalk, thereby blocking the rotation of the latter and preventing ATP hydrolysis[19]. This inhibitory mechanism has recently been shown to be highly dependent on the catalytic activity of the enzyme[20]. For extensive reviews on the structure and rotary mechanism of ATP synthase[21-23].

Given its essential role in ATP synthesis, defects in ATP synthase are involved in extremely severe and often lethal diseases because of the marked reduction in ATP production and concomitant accumulation of reactive oxygen species (ROS)[24,25]. Such pathologies are mostly linked to mutations in ATP synthase subunits, the most frequently described being mapped to the mitochondrial ATP6 gene (encoding for subunit a) which is responsible for the maternal-inherited NARP syndrome (Neurogenic muscle weakness, Ataxia, Retinitis Pigmentosa) or Leigh syndrome (severe and fatal encephalopathy), depending on the extent of heteroplasmic mutations. Defects in ATP synthase biogenesis, due to mutations in genes encoding its assembly factors for instance, have also been described[26]. Finally, abnormal accumulations of ATP synthase subunits have been found in various neurodegenerative diseases, such as subunit c accumulation in neuronal ceroid lipofuscinosis[27,28], or subunit α accumulation in Alzheimer's disease[29].

Over the past few years, various reports by many independent groups have described the presence, at the cell surface of mammalian cells, of various subunits of ATP synthase, which will also be referred to as ecto-F_1-ATPase throughout this review. The exact structure of this ectopically-expressed ATP synthase and whether it is exactly the same enzyme as the mitochondrial complex have not been definitely determined. Nevertheless, a number of pieces of experimental evidence suggest that it is a closely related entity. We will review here the various reports describing the unexpected cell surface expression of ATP synthase and its possible functions.

IS ECTO-F_1-ATPASE THE SAME ENTITY AS MITOCHONDRIAL ATP SYNTHASE?

Over the past few years, a growing number of reports, including our work[30,31], have described the presence of ATP synthase subunits at the surface of a wide variety of tumor as well as normal cells by fluorocytometry.

While most of these reports focused on the α and β subunits against which monoclonal antibodies are available[30-32], a few have described the presence of other subunits at the cell surface by using in-house manufactured polyclonal reagents analyzed by cytometry or confocal microscopy. Vantourout *et al*[33] have shown that high cell surface expression of major histocompatibility complex class I (MHC- I) antigens interferes with the detection

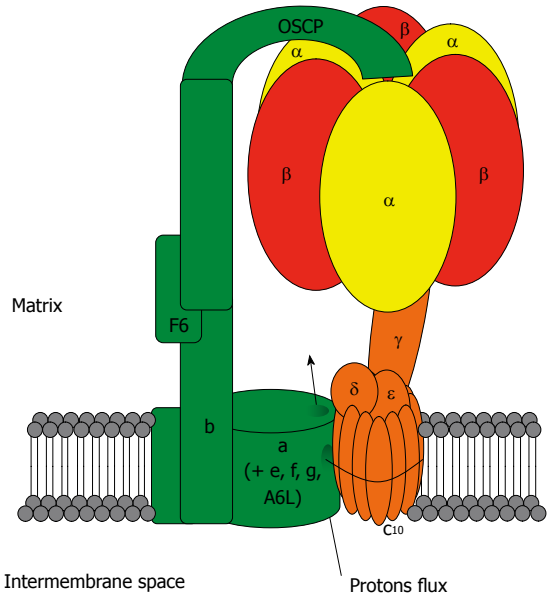

Figure 1 Mitochondrial ATP synthase. In eukaryotic cells, F_1F_o ATP synthase is a complex molecular motor composed of 16 unique subunits (α, β and c being present in multiple copies in the whole complex). It comprises the activity-bearing subunits (three αβ heterodimers), a rotor (in orange: subunits γ, δ, ε and the c ring) and a stator (in green: subunits a, e, f, g, A6L, b, F6, d and OSCP) which holds the αβ heterodimers. It is organized into two major domains: a soluble F_1 domain (α, β, γ, δ, ε) and a membrane-associated F_o domain. The F_1 domain bears the catalytic activity while the F_o domain is a proton channel. The F_o domain uses the proton gradient created by the four other complexes of the respiratory chain to drive the rotation of the central stalk, which will alternatively change the conformation of the three αβ heterodimers, leading to the synthesis of ATP from ADP and Pi. In the case of the eukaryotic ATP synthase, the translocation of 10 protons through the c ring will lead to a full turn, and the synthesis of 3 ATP molecules. In the absence of a proton gradient, or if the F_1 domain is isolated from the F_o domain, the enzyme behaves as an ATP hydrolase.

Table 1 Ecto-F_1-ATPase subunit detection in various cell types

Cells/tissues	Subunits	Methods	Ref.
K562, A549, Raji	β	B, FC, S	[38]
HUVEC	α, β, IF1	B, FC, CM	[35,39,40]
HepG2, IHH, Human hepatocytes	α, β	SPR, FC, CM	[30]
3T3-L1	α, β	B, CM	[41]
Jurkat	β	RP + MS	[42]
Rat hepatocytes	α, β, δ, γ, b, d, e, F6, OSCP	CM	[37]
HaCat, Human skin	β	RP + MS, CM	[43]
Daudi, RPMI-8226, U937, SK-NEP, G401, G402	α and/or β	FC	[31]
Awells, ST-Emo, HeLa, fibroblasts, Rma-S	α, β	B, CM, FC	[33]
Rat tonsils	β	PM	[44]
Neurons, neuroblastoma, astrocytoma	α	B, CM	[45]
Osteosarcoma	α, β, d	CM	[36]

Studies reporting a cell surface expression of ATP synthase subunits are summarized. The experimental approaches used in these studies are mentioned. B: Cell surface biotinylation; FC: Flow cytometry; S: Sequencing; CM: Confocal microscopy; SPR: Surface plasmon resonance (Biacore); RP + MS: Rafts purification and mass spectrometry; PM: Plasma membrane extraction. Cell lines/tissues are of human origin unless otherwise noted. K562: Erythroleukemia; A549: Lung adenocarcinoma; RPMI-8226: B myeloma; Raji, Daudi: Burkitt lymphomas; Jurkat: T lymphoma; HUVEC: Human umbilical vein endothelial cells; HepG2: Hepatocarcinoma; IHH: Immortalized human hepatocytes; 3T3-L1: Murine fibroblasts (differentiated into adipocytes); HaCat: Immortalized human keratinocytes; U937: Monocytic leukemia; SK-NEP, G401, G402: Renal cell carcinomas; Awells, ST-EMO: EBV-immortalized B cells (ST-EMO was derived from a major histocompatibility complex class I deficient patient); HeLa: Cervix carcinoma; Rma-S: Murine T lymphoma.

of the α and β subunits using some monoclonal reagents, probably through molecular hindrance. In a recent work, Mangiullo *et al*[34] described the presence of FoI-PVP (b subunit), γ and OSCP subunits at the cell surface of rat hepatocytes. The inhibitory protein IF_1 has also been shown to be present at the cell surface[35]. Recently, d and OSCP subunits were shown to be expressed on the surface of osteosarcoma cells[36]. In a number of studies, biochemical approaches have been used to confirm the presence of F_1-ATPase/ATP synthase in plasma membranes. Bae *et al*[37] have shown *via* a proteomics approach that multiple ATP synthase subunits are found in the lipid rafts of rat hepatocytes and $HepG_2$ cells, a human hepatocarcinoma cell line. F_1-ATPase components have been coprecipitated with cell surface proteins from hemopoietic tumors by Vantourout *et al*[33]. One could argue that pure plasma membrane preparations are extremely difficult to obtain and easily contaminated by membranes originating from different sub-cellular compartments. A very low amount of mitochondrial material could explain the presence of ATP synthase subunits in membrane preparations. Nevertheless, in some works using biochemical approaches[33,34] the absence of other mitochondrial proteins in samples has been carefully checked, ruling out a contamination by mitochondrial material. The observation of multiple components of F_1-ATPase by cytometry, and the identification of some of them by mass spectrometry and peptide sequencing[30,37,38], make very unlikely the hypothesis that ecto-F_1-ATPase detection could be due to cross-reactivity with other proteins bearing sequence similarities.

The presence of subunits other than α and β is also not completely unexpected. Indeed, since both these subunits do not have any membrane-anchoring domain, the cell surface expression of other subunits, especially from the F_o domain, would be required to allow such a localization of the complex.

To summarize, while definitive proof may still be lacking, it seems now highly probable that ecto-F_1-ATPase is a cell surface complex identical or closely related to the mitochondrial ATP synthase. The wide range of cellular types that have been shown to express ecto-F_1-ATPase (Table 1) certainly suggest that this ectopic expression is more likely to be a ubiquitous phenomenon rather than restricted to a few cell lines. Among all samples that we have analyzed for ecto-F_1-ATPase expression using different approaches, so far only one, namely 721.221 (an EBV-immortalized B cell line), seemed to not express ecto-F_1-ATPase, or at extremely low levels that are not detectable[33,46].

AN UNEXPECTED AND SO FAR UNEXPLAINED LOCALIZATION

While many proteins are now known to have multiple sub-cellular localizations, a cell surface expression of mitochondrial proteins is quite unusual and puzzling. To our knowledge, there is so far no experimental work precisely deciphering the mechanism used by ATP synthase to reach the plasma membrane (PM).

In our view, two main hypotheses are conceivable: (1) ATP synthase subunits are routed to the PM instead of the mitochondria. The concept of multiple localization of a given protein is quite established now and several mechanisms can be involved (see Karniely *et al*[47] for an extensive review). The first is that different mRNA isoforms of ATP synthase subunits could hold a cell-surface signal peptide rather than mitochondrial targeting signal. Bovine subunit c has been reported to have two isoforms (encoded by two different genes) with slightly different signal peptides[48]. However, to our knowledge, this is an isolated case so far, and there is no experimental evidence suggesting that these different peptides lead to different localizations. Another explanation is that the known signal peptides of ATP synthase subunits are ambiguous and could lead to a mitochondrial or cell surface expression. We have analyzed some of ATP synthase subunit leader peptides with the SignalP software[49] and some leader peptides of ATP synthase subunits may share some predicted similarities with PM-targeting sequences. Finally, some proteins use two targeting signals on the same polypeptide to reach different destinations, such as Catalase A in yeast[50]. This latter possibility seems unlikely since no PM signal can be identified in the sequences of ATP synthase subunits. Even if this first hypothesis cannot be definitely ruled out yet, it is in our opinion the less likely for a number of reasons. Firstly, it would imply that all subunits display ambiguous signals, or have different isoforms, and so far there is no formal proof of this. Secondly, two subunits (namely A6L and a) are encoded by the mitochondrial genome, and it seems unlikely that they would be targeted to the cell surface instead of staying in the mitochondria. Finally, the ATP synthase assembly factors would also have to be expressed elsewhere [such as the endoplasmic reticulum (ER)] to allow the expression of a complete and correctly assembled complex at the cell surface; and (2) The simpler and thus more tempting hypothesis is that once assembled into the mitochondria, ATP synthase then reaches the cell surface. As a direct rerouting of the whole complex *via* transporters is improbable in this case, given the size of ATP synthase (around 600 kDa and a dimension of 19 nm × 11 nm), it is suggested that the complex reaches the cell surface *via* vesicular transport or fusion of mitochondrial membranes with the PM.

In healthy cells, mitochondria form a dynamic network and constantly divide and fuse, a process which is believed to maintain the integrity and quality of the mitochondrial function and implicates several recently identified proteins such as mitofusins or Drp1[51,52]. A direct fusion of mitochondrial membranes with the PM, resulting in a cell surface expression of ATP synthase, seems difficult to imagine mostly because mitochondria have two membranes and ATP synthase is located in the inner one. Unless the fusion occurs between PM and mitochondrial contact sites, which are distinct structures playing an important role in the mitochondrial metabolism and apoptosis[53], this hypothesis is unlikely.

However, there are now several lines of evidence indicating that mitochondria communicate with other cellular compartments. A recent report by de Brito *et al*[54] has shown that mitofusin 2 is implicated in the interaction between ER and mitochondria, allowing the exchange of Ca^{2+} between those organelles. In another recent report, Neuspiel *et al*[55] identified a new outer membrane mitochondria-anchored protein ligase named MAPL (mitochondria-anchored protein ligase) and characterized the sorting of MAPL into mitochondria-derived vesicles which then fuse with peroxisomes. Interestingly, Neuspiel *et al*[55] have also identified distinct vesicles containing another mitochondrial protein, Tom20 (translocase of outer membrane 20, a member of the complex importing proteins encoded by the nuclear genome into mitochondria). These Tom20-containing vesicles do not fuse with peroxisomes and their fate is currently under investigation. In addition, Soltys *et al*[56] have shown that Hsp60, a mitochondrial chaperone, can be found in vesicles that are distinct from major organelles and may communicate with the PM. Interestingly, Hsp60 has also been shown to be expressed at the cell surface[57,58]. In both cases, these uncharacterized vesicles could thus be part of a shuttle system between mitochondria and PM, allowing the cell surface expression of selected mitochondrial proteins. In this context, Rab32 or endophilin B1, involved in mitochondrial dynamics and morphology, respectively[59,60], might also be involved in the biogenesis of mitochondria-derived vesicles and the trafficking towards the cell surface, raising the question of the existence of a new intracellular pathway from mitochondria to cell surface responsible for the targeting of the F_1-ATPase.

A recent report has shown that a deficiency in palmitoyl protein thioesterase 1 (Ppt1), responsible for the infantile neuronal ceroid lipofuscinosis syndrome, was linked with an increase in ecto-F_1-ATPase expression in neurons[61]. This suggests that Ppt1 might be involved in the regulation of the subcellular localization of ATP synthase. In addition, Schmidt *et al*[45] have immunoprecipitated F_1-ATPase α chain from the synaptic membranes of murine neural cells. Strikingly, they reported that this subunit was at least in part N-glycosylated and that this post-translational modification was abrogated by treating cells with the Golgi-disrupting drug brefeldin A. They also identified a unique N-glycosylation site which is absent in the human α-subunit sequence. This suggests that at least some components of the mitochondrial F_1-ATPase can transit through the Golgi apparatus, although the mechanism is still enigmatic.

Another question regarding the cell surface expres-

sion of ATP synthase is its sub-localization at the PM. Some of its subunits, as well as a few other mitochondrial proteins, have often been found in lipid rafts in proteomic studies[37,42]. However, a recent report by Zheng *et al*[62] suggested that mitochondrial proteins, including ATP synthase subunits, were the best contaminants of raft preparations, mostly based on the fact that they were resistant to cholesterol depletion by methyl-β-cyclodextrin, whereas raft-resident proteins usually are sensitive to this treatment. Detection of ecto-F_1-ATPase by confocal microscopy usually reveals a patchy pattern[30,33], which is reminiscent of several proteins residing in lipid rafts. We also have experimental evidence showing at least a partial co-localization of ecto-F_1-ATPase subunits with the raft markers cholera toxin (unpublished data). This may indicate that the presence of ecto-F_1-ATPase in lipid rafts is not exclusive. It may also vary with the cell line or conditions of culture. Similarly, its detection in isolated rafts may depend on raft purification procedures. Thus, more extensive studies are required to definitively answer this question.

A NEW LOCALIZATION FOR MANY NEW ROLES: ECTO-F_1-ATPASE AS A MOONLIGHTING PROTEIN

The term "moonlighting protein" was introduced in 1999 by Jeffery[63] to describe proteins that could perform several unrelated roles. Their different functions can depend on different localizations, expression in different cell lines, ligand concentration, or on many other mechanisms.

ATP synthase appears as a typical example of a moonlighting protein. Its α subunit, for instance, has been shown to be part of a complex importing cytosolic tRNAs into mitochondria in *Leishmania*[64]. Subunit F6 has also been shown to be present in plasma and involved in hypertension through its binding on ecto-F_1-ATPase expressed on endothelial cells[65]. It is also now clear that the whole complex expressed at the cell surface is implicated in many physiological roles, depending on the ligand and the cell type (Table 2). So far, the most documented roles of ecto-F_1-ATPase are its involvement in HDL endocytosis by hepatocytes, endothelial cell survival, and as an immunological ligand for a subset of non-conventional T lymphocytes (Figure 2).

Hepatic HDL-cholesterol uptake

Our work on high density lipoproteins (HDL) has identified ecto-F_1-ATPase as the receptor of apolipoprotein A-I (apoA-I), the major protein component of HDL. Indeed, binding of HDL on hepatocyte membranes displayed two binding sites with high and low affinities[68]. A high-affinity receptor for lipid-free apoA-I was purified by Biacore and identified as the β-chain of ATP synthase. As binding of lipid-free apoA-I to ecto-F_1-ATPase activated endocytosis of whole and fully lipidated HDL by the low-affinity binding sites, we have hypothesized that

Table 2 Ligands and roles of Ecto-F_1-ATPase

Cells/tissues	Ligands	Proposed roles	Ref.
K562, A549, Raji	?	NK/LAK-mediated tumor cell lysis	[38]
HUVEC	Angiostatin	Cell survival through ATP production	[40,60,66]
	HDL/apoA-I	Cell survival through purinergic receptor activation	
	FC6	Control of blood pressure	
Hepatocytes	HDL/apoA-I	HDL endocytosis	[30]
3T3-L1, HaCat	?	Cell survival	[41,43]
Tumor cells	Vγ9/Vδ2 TCR, apoA-I, MHC- I	Tumor cell recognition by Vγ9/Vδ2 T cells/presentation of phosphoantigens	[31,33,46,67]
Tonsils (mouse)	Enterostatin	Food intake regulation	[44]
Neurons	APP, Amyloid β-peptide	Synaptic plasticity regulation	[45]

Major roles of Ecto-F_1-ATPase described in the literature, as well as ligands involved, are summarized here. MHC- I : Major histocompatibility complex class I ; NK: Natural killer; LAK: Lymphokine activated killer; HDL: High density lipoprotein; apoA-I: Apolipoprotein A-I; FC6: Coupling factor 6; ATP: Adenosine triphosphate; APP: Amyloid precursor protein. Cells/tissues are of human origin unless otherwise noted. K562: Erythroleukemia; A549: Lung adenocarcinoma; Raji: Burkitt's lymphoma; HUVEC: Human umbilical vein endothelial cells; 3T3-L1: Murine fibroblasts (differentiated into adipocytes); HaCat: Immortalized human keratinocytes.

enzymatic activity of ecto-F_1-ATPase might play a role in this stimulatory process. Indeed, direct addition of ADP increased HDL endocytosis. We showed that binding of apoA-I on ecto-F_1-ATPase increased its hydrolase activity and led to an enhanced HDL endocytosis. Later, we identified the purinergic $P2Y_{13}$ receptor as the target of ADP produced by ecto-F_1-ATPase[69] and we have shown recently that the small GTPase RhoA and its effector ROCK I were activated downstream of $P2Y_{13}$[70]. We could demonstrate that this F_1-ATPase/$P2Y_{13}$-mediated HDL endocytosis pathway is controlled through cell surface adenylate kinase (AK) activity which consumes ADP generated upon activation of ecto-F_1-ATPase by apoA-I and therefore constitutively inhibits $P2Y_{13}$ and subsequent hepatic HDL endocytosis[71]. In addition, niacin (vitamin B3), which is well-known to increase plasma HDL levels in humans, has recently been shown to inhibit cell surface expression of ecto-F_1-ATPase in hepatocytes[72]. This study thus suggests the implication of ecto-F_1-ATPase in HDL metabolism.

Our recent work has confirmed the role of the $P2Y_{13}$ ADP-receptor in HDL-mediated reverse cholesterol transport (RCT) *in vivo*[73]. We showed that $P2Y_{13}$-deficient mice exhibited a decrease in hepatic HDL cholesterol uptake, hepatic cholesterol content, and biliary cholesterol output, although their plasma HDL and other lipid levels were normal. These changes translated into a substantial decrease in the rate of macrophage-to-feces RCT. Therefore, hallmark features of RCT are impaired in $P2Y_{13}$-deficient mice. Furthermore, cangrelor, a partial agonist of $P2Y_{13}$, stimulated hepatic HDL uptake and biliary lipid

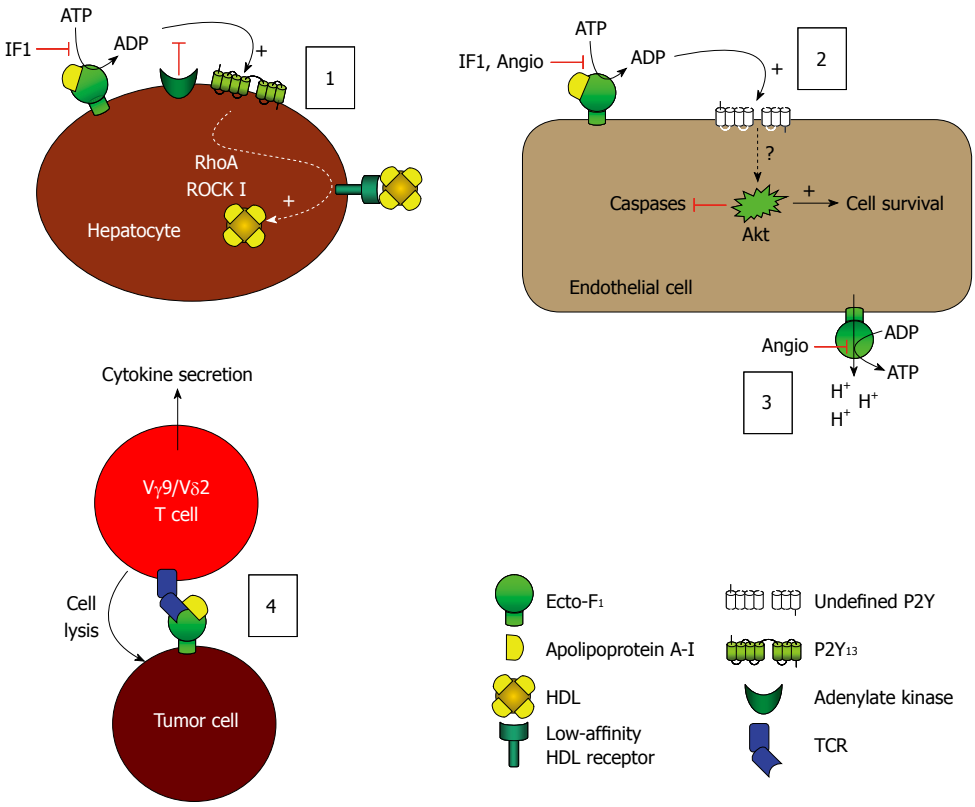

Figure 2 Model of the leading hypotheses regarding the distinct cellular events mediated by ecto-F$_1$-ATPase. (1) Model of F$_1$-ATPase/P2Y$_{13}$-mediated high density lipoproteins (HDL) endocytosis by hepatocytes. ApoA-I binding to ecto-F$_1$-ATPase stimulates extracellular ATP hydrolysis, and the ADP generated selectively activates the nucleotide receptor P2Y$_{13}$ and subsequent RhoA/ROCK I signaling that controls holo-HDL particle endocytosis. Conversely IF$_1$ inhibitor which inhibits ecto-F$_1$-ATPase activity or adenylate kinase (AK) activity which consumes ADP generated upon activation of ecto-F$_1$-ATPase, downregulates holo-HDL particle endocytosis. Thus, HDL endocytosis depends on the balance between the AK activity that removes extracellular ADP and the ecto-F$_1$-ATPase that synthesizes ADP. Activation of ADP synthesis by apoA-I unbalances the pathway and increases HDL endocytosis when required; (2), (3): Model of F$_1$-ATPase-mediated endothelial cell protection. (2): Hydrolytic activity of ecto-F$_1$-ATPase expressed on endothelial cells can be increased by apoA-I, stimulating cell proliferation. Conversely, inhibition by IF$_1$, angiostatin increases apoptosis and blocks proliferation both at the basal level and after stimulation by apoA-I. The downstream receptors and intracellular signaling are still not completely characterized, but ADP-sensitive receptors could be involved; (3): The anti-angiogenic activity of angiostatin is attributed to its ability to inhibit ATP production by ecto-F$_1$-ATPase, thus eliminating a significant source of energy and reducing proliferation. It is also proposed that this inhibition prevents the extrusion of protons by ecto-F$_1$-ATPase, thus lowering intracellular pH and hampering cell survival; and (4) Model of F$_1$-ATPase-mediated tumor cell recognition by Vγ9/Vδ2 T lymphocytes. Ecto-F$_1$-ATPase is found at the surface of tumor cells and plays a role in the presentation of adenylated derivatives of small phosphoantigens, which are classical inducers of cell lysis by Vγ9/Vδ2 T lymphocytes. Recognition of ecto-F$_1$-ATPase by the γ9/δ2 TCR is stimulated by apoA-I and requires a low expression of class I HLA-antigens, a situation commonly found in tumor cells.

secretions in normal mice and in mice with a targeted deletion of scavenger receptor class B type I (SR-BI) in liver [hypomSR-BI-knockout(liver)] but had no effect in P2Y$_{13}$ knockout mice, which indicate that the P2Y$_{13}$-mediated HDL uptake pathway is independent of SR-BI-mediated HDL selective cholesteryl ester uptake. Pharmacological activation of the P2Y$_{13}$ receptor might thus represent a new therapeutic approach to improve HDL-cholesterol removal and/or turnover, which could be an alternative way to prevent and treat atherosclerosis.

Endothelial cell survival

Ecto-F$_1$-ATPase has also been characterized by Moser *et al*[32] as the receptor of angiostatin, an inhibitor of angiogenesis originating from the proteolysis of plasminogen. Later work established that ecto-F$_1$-ATPase was able to synthesize ATP at the cell surface of endothelial cells. This ATP production would facilitate the proliferation of these cells, especially under hypoxic conditions which are known to impair cell survival through the inhibition of oxidative phosphorylation. The anti-angiogenic activity of angiostatin is attributed to its ability to inhibit ATP production by ecto-F$_1$-ATPase, thus eliminating a significant source of energy and reducing proliferation. It was also proposed that this inhibition prevents the extrusion of protons by ecto-F$_1$-ATPase, thus lowering intracellular pH and hampering cell survival[39,66].

It is well known that HDL and apoA-I have a protective effect against the development of endothelial dysfunction, one of the first events in the pathogenesis of atherosclerosis, by enhancing endothelial cell survival. The mechanisms mediating this atheroprotection are not well understood, but interactions between HDL and apoA-I with cell surface receptors have been proposed to be essential contributors. Considering that both HDL and apoA-I bind ecto-F$_1$-ATPase, we have evaluated the hypothesis that the protective effects of apoA-I on endothelial cells are dependent on ecto-F$_1$-ATPase activity. This hypothesis

is also supported by the anti-angiogenic effect of angiostatin.

As shown for hepatocytes[30], the hydrolytic activity of ecto-F_1-ATPase expressed on endothelial cells can be increased by apoA-I, stimulating cell proliferation and inhibiting apoptosis in a dose-dependent manner[40]. Conversely, inhibition by IF_1, angiostatin or antibodies directed against the β-chain of ecto-F_1-ATPase increases apoptosis and blocks proliferation both at the basal level and after stimulation by apoA-I. These results are in favor of a direct effect of the ecto-F_1-ATPase activity on endothelial cell survival through extracellular ADP production. The downstream receptors and intracellular signaling are still not completely characterized, but ADP-sensitive receptors could be involved.

This pathway thus provides a new mechanism of action of apoA-I in endothelial protection distinct from other HDL-mediated protective effects previously described[74]. Thus, depending on the cell type, distinct pathways seem to be involved downstream of F_1-ATPase activation by apoA-I and induce distinct cellular events such as stimulation of HDL uptake by hepatocytes or endothelial cell survival.

Tumor cell recognition

Vγ9/Vδ2 T lymphocytes are a non-conventional subpopulation of cytotoxic T cells unique to humans and higher primates. These cells are known to participate in the immune response against various intracellular pathogens as well as tumor cells, through the recognition of small alkyl diphosphate molecules termed phosphoantigens[75,76]. Experimental evidence suggests that recognition of these antigens requires a cell-cell contact, and thus may depend on a cell surface presentation molecule expressed on target cells but distinct from classical antigen presentation molecules such as major histocompatibility antigens and CD1[77]. This is confirmed by recent data showing that the recognition of phosphoantigens by a soluble recombinant Vγ9/Vδ2 T cell receptor (TCR) on the cell surface is dependent on a trypsin-sensitive structure[78]. We have initially identified apoA-I as an enhancer of the Vγ9/Vδ2 T cell response, and shown that these cells can actually recognize F_1-ATPase on the surface of tumor cells through their TCR[31]. We have also shown that ecto-F_1-ATPase interacts with MHC- I molecules on the cell surface of various cell lines, leading to a masking of epitopes which prevents the detection of ecto-F_1-ATPase by commercially available antibodies[33]. This suggests that cell surface expression of ATP synthase cannot be formally ruled out simply because it is not detected by flow cytometry or confocal microscopy. It is also of particular interest for the physiology of Vγ9/Vδ2 cells because, like natural killer cells, they express inhibitory receptors which bind MHC antigens and modulate TCR signaling and lymphocyte activation[79].

Our latest work has focused on the study of a novel ATP analog, ApppI [triphosphoric acid 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester] in which the γ-phosphate of ATP is esterified with isopentenol. This metabolite has recently been identified in cells treated with aminobisphosphonate drugs which are known to potently sensitize cells to Vγ9/Vδ2 T cell cytotoxicity. As it is an ATP analog and has a structure reminiscent of nucleotidic phosphoantigens naturally produced by some bacteria, we thus used this molecule to explore the potential role of ecto-F_1-ATPase in phosphoantigen presentation to Vγ9/Vδ2 T cells. We found that classical targets of Vγ9/Vδ2 T cells can spontaneously produce ApppI[67]. Furthermore, it stably binds to purified ATP synthase and ApppI-loaded ATP-synthase efficiently activates Vγ9/Vδ2 T cells *in vitro*. Our current model proposes that phosphoantigens produced by tumor cells or intracellular pathogens are converted to nucleotidic derivatives, loaded onto ATP synthase, and presented to Vγ9/Vδ2 T cells when the complex is exported to the cell surface[46].

As ATP synthase is present at the cell surface of a number of normal cells, the potential self-reactivity of Vγ9/Vδ2 T cells is probably tightly controlled by the interaction between ecto-F_1-ATPase and MHC- I , as well as by phosphoantigen production by the target cell. Moreover, these observations suggest another beneficial role for apoA-I; as an immunomodulator in anti-tumor responses.

Since ecto-F_1-ATPase has already been detected in a wide variety of cells, with many different ligands, there is no doubt that it is probably implicated in many other cellular processes. In many instances, these roles seem to rely on the enzymatic activity of ATP synthase. While its hydrolase activity is undoubtedly possible since it does not need a proton flux nor requires a fully assembled F_1F_o complex, there are still conflicting reports regarding the ability of the cell surface complex to synthesize ATP.

TO SYNTHESIZE OR NOT TO SYNTHESIZE, THAT IS THE QUESTION

The first work showing that ecto-F_1-ATPase can synthesize ATP from ADP and Pi was reported in endothelial cells[39] and showed that angiostatin was able to inhibit both the synthase and hydrolase activities of ATP synthase. This was shown by bioluminescence measurement of ATP and a radioactive thin layer chromatography (TLC) assay using [^{3}H]ADP and ^{32}Pi. In later work, the authors have shown that ATP production was enhanced when extracellular pH was low, hypothesizing that concomitantly with ATP synthesis, ecto-F_1-ATPase would extrude protons from the cytoplasm. It was also proposed that this process improves the resistance of tumor and endothelial cells to low pH. Similarly, it would also improve cell survival in hypoxic conditions, a hallmark of tumoral microenvironment. Since then, a number of independent studies have reported a synthase activity in many different cell types, including keratinocytes, adipocytes, or hepatocytes, among others[34,43,41]. Most of these works have used a bioluminescence assay to measure the production of ATP after addition of ADP in the culture media and underlined

the importance of ecto-F_1-ATPase in this production through the use of oligomycin, a well known inhibitor of mitochondrial ATP synthase.

However, whether or not ecto-F_1-ATPase can synthesize ATP is still debated, as there are several conflicting reports. Several studies by our group and others seem to completely rule out a potential contribution of ecto-F_1-ATPase to extracellular conversion of ADP to ATP. Yegutkin *et al*[80] have shown that ATP production after addition of ADP to endothelial cells was not increased in the presence of Pi, which should be the case if ecto-F_1-ATPase was able to synthesize ATP. They concluded that ADP phosphorylation was mostly due to cell surface adenylate kinase (AK, which produces ATP and AMP from two ADP molecules) and/or nucleotide diphosphokinase (NDPK, which transfers terminal phosphate from a nucleotide triphosphate to a nucleotide diphosphate) enzymatic activities. This is in agreement with our results in hepatocytes as well as in endothelial cells. Indeed, we have observed that addition of [^{3}H]ADP and ^{32}Pi to the cell medium does not lead to production of dual-labeled ATP, but only [^{3}H]ATP[30,40,71], indicating that free inorganic phosphate is not used for ATP synthesis and excluding a role for ATP synthase in the process of ATP generation.

Most of the studies reporting a synthase activity have shown that oligomycin decreased extracellular ATP production after addition of ADP to culture media, thus suggesting that ecto-F_1-ATPase was indeed able to synthesize ATP. However, in our hands, oligomycin had no effect on ATP synthesis on the cell surface of human hepatocytes after addition of ADP[71]. The reason for these discrepancies is not clear. One should note that oligomycin has been shown to inhibit other enzymes, such as the non-gastric H,K-ATPase[81]. Thus it does not appear to be a strictly specific inhibitor of ATP synthase/F_1-ATPase and could act on other nucleotide-converting enzymes. One cannot exclude also that the activity of ecto-F_1-ATPase depends on particular experimental settings (culture conditions, cell type) and on the metabolic status of the cells. Nevertheless, in mitochondria, ATP production by ATP synthase is strictly dependent on the electrochemical proton gradient between the two sides of the inner mitochondrial membrane. This gives the chemical energy required for ATP synthesis. Whether a strong proton flux can occur at the cell surface and lead to ATP synthesis by ecto-F_1-ATPase is not certain and in the case of extracellular acidosis, the proton gradient would not be favorable for ATP synthesis. Mangiullo *et al*[34] have recently shown that ecto-F_1-ATPase was active in ATP synthesis and that it was correlated to an oligomycin-sensitive proton flux from the cytoplasm to the extracellular medium in the case of intracellular acidosis. Although, as discussed above, oligomycin might inhibit other proton pumps, this suggests that synthesis might occur in particular metabolic settings. It remains to be demonstrated whether these experimental conditions are relevant to physiological situations. Thus, the actual role of ecto-F_1-ATPase in proton transport across the PM remains arguable.

Accordingly, the synthase activity of ecto-F_1-ATPase is still an open issue, and there certainly is a need for reassessment of results obtained with the various techniques assessing ATP synthase activity as well as with the different inhibitors.

CONCLUSION

Over the past few years, data originating from many different groups have confirmed the cell surface expression of this mitochondrial enzyme. While this ectopic expression is now certainly widely accepted, there is still a need for a definitive characterization of its enzymatic activity, as well as new insights into the mechanism of its cell surface targeting.

In our opinion, the synthase activity of ecto-F_1-ATPase is still heavily challenged on both experimental and theoretical aspects. It is an important issue that requires additional work and may depend on the development of novel techniques which would be more specific and sensitive than those currently used. Nevertheless, the monitoring of dual-labeled ATP production from [^{3}H]ADP and ^{32}Pi by high-performance liquid chromatography (HPLC) is probably so far the most reliable method to determine whether ecto-F_1-ATPase is active in ATP synthesis, since ATP synthase is the only known enzyme able to generate ATP directly from ADP and inorganic free phosphate. Therefore, it should be the method of choice to study the enzymatic properties of ecto-F_1-ATPase.

Understanding how ecto-F_1-ATPase reaches the cell surface is a challenging but extremely important point. As the use of siRNAs targeting ATP synthase subunits is hardly conceivable since it will undoubtedly rapidly lead to cell death, inhibiting its cell surface expression might help to better characterize its roles. Further studies are definitely required to decipher this mechanism.

ATP synthase is a fascinating molecular machine and a perfect example of a nanomotor. It has been one of the most studied enzymes over the past 40 years and garnered Professor Boyer PD and Sir Walker JE the Nobel Prize in Chemistry in 1997 for their outstanding work on the comprehension of its structure and the ATP synthesis mechanism[82-84]. Its central role in energy production is fundamental, an active person synthesizing their own weight in ATP every day. This essential role is now enhanced by its new implication in various important cellular processes, achieved through this ectopic localization and interactions with diverse ligands. We are only beginning to decipher these new functions.

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TOPIC HIGHLIGHT

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Biliary cholesterol secretion: More than a simple ABC

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Abstract

Biliary cholesterol secretion is a process important for 2 major disease complexes, atherosclerotic cardiovascular disease and cholesterol gallstone disease. With respect to cardiovascular disease, biliary cholesterol secretion is regarded as the final step for the elimination of cholesterol originating from cholesterol-laden macrophage foam cells in the vessel wall in a pathway named reverse cholesterol transport. On the other hand, cholesterol hypersecretion into the bile is considered the main pathophysiological determinant of cholesterol gallstone formation. This review summarizes current knowledge on the origins of cholesterol secreted into the bile as well as the relevant processes and transporters involved. Next to the established ATP-binding cassette (ABC) transporters mediating the biliary secretion of bile acids (ABCB11), phospholipids (ABCB4) and cholesterol (ABCG5/G8), special attention is given to emerging proteins that modulate or mediate biliary cholesterol secretion. In this regard, the potential impact of the phosphatidylserine flippase ATPase class I type 8B member 1, the Niemann Pick C1-like protein 1 that mediates cholesterol absorption and the high density lipoprotein cholesterol uptake receptor, scavenger receptor class B type I, is discussed.

Key words: Cholesterol; Bile; Gallstone; Atherosclerosis; Reverse cholesterol transport; Lipoproteins; High density lipoprotein; Scavenger receptor class B type I

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INTRODUCTION: WHAT IS THE IMPORTANCE OF BILIARY CHOLESTEROL SECRETION?

The liver plays a central role in cholesterol metabolism (Figure 1). Hepatocytes not only express a number of different lipoprotein receptors including low density lipoprotein receptor (LDLR), LDLR-related protein (LRP) and scavenger receptor class B type I (SR-BI), that enable them to take up cholesterol from virtually all lipoprotein subclasses, but cholesterol is also synthesized *de novo* within the liver in a regulated fashion[1]. In addition to these input pathways into the hepatic cholesterol pool, the liver is equipped to actively secrete cholesterol *via* 2 different routes: (1) within triglyceride-rich very low density lipoproteins (VLDL)[2,3], thereby supplying peripheral cells

with fatty acids, fat soluble vitamins and cholesterol; (2) by secretion into the bile either directly as free cholesterol or after conversion into bile acids, thereby providing a means of irreversible elimination of cholesterol from the body *via* the feces[4,5]. In general, the different hepatic cholesterol fluxes are interrelated, however, some are also markedly separated, as will be discussed later in this review.

Biliary cholesterol secretion itself is directly linked to 2 major disease complexes with a high relevance for health care systems worldwide, namely atherosclerotic cardiovascular disease (CVD) and gallstone disease. In atherosclerotic CVD, biliary cholesterol secretion is considered the final step in the completion of the reverse cholesterol transport (RCT) pathway[6,7]. The term RCT comprises the transport of peripheral cholesterol back to the liver for excretion into bile, most importantly cholesterol accumulating within macrophage foam cells in atherosclerotic lesions[8]. For RCT an enhanced biliary secretion of cholesterol originating from peripheral pools relevant for CVD is desirable. On the other hand, increased biliary cholesterol secretion is related to biliary cholesterol supersaturation, which is an important determinant for the formation of cholesterol gallstones that constitute more than 90% of all gallstones[9,10]. Notably, both CVD[11,12] and gallstone disease[13] also have a strong inflammatory component that plays an important role in the pathogenesis of these diseases. However, an in-depth understanding of the metabolic processes and transporters involved in the regulation of biliary cholesterol secretion is important and might conceivably reveal relevant targets for the treatment of CVD as well as cholesterol gallstone disease.

WHAT IS THE ORIGIN OF CHOLESTEROL SECRETED INTO BILE?

The most relevant source of cholesterol secreted into the bile is cholesterol derived from plasma lipoproteins, and a less relevant source is cholesterol originating from *de novo* synthesis or hydrolysis of stored cholesteryl ester[14]. High density lipoprotein (HDL) appears to be the preferential contributor for cholesterol secreted into bile[15]. In humans with a bile fistula, cholesterol originating from HDL appeared more rapidly in bile compared with LDL cholesterol[16]. Additional evidence for a more prominent role of HDL over LDL came from experiments demonstrating that biliary cholesterol secretion remained essentially unchanged when plasma LDL cholesterol levels specifically were reduced by 26% by means of LDL apheresis[17]. In contrast, reduction in plasma LDL cholesterol resulted in a consecutive decrease in biliary bile acid secretion[17], thereby lending further experimental evidence to older literature suggesting a metabolic compartmentalization of hepatic cholesterol pools with regard to bile acid synthesis *vs* direct biliary secretion[18,19]. However, definitive studies exploring the underlying metabolic pathways are still lacking.

It is, however, important to note, that modulation of plasma HDL cholesterol levels does not influence mass biliary cholesterol secretion, since ATP-binding cassette transporter A1 (ABCA1) knockout mice[20] as well as apolipoprotein-A-I (apoA-I) knockout mice[21,22] display unaltered biliary cholesterol secretion rates. These data indicate that specific intrahepatic metabolic and transport processes are most relevant and rate-limiting for biliary secretion of cholesterol.

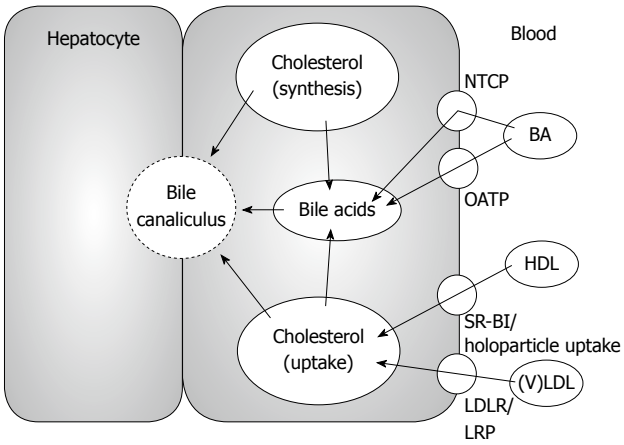

Figure 1 Overview of hepatic cholesterol metabolism in relation to biliary sterol secretion. HDL: High-density lipoprotein; BA: Bile acid; LDLR: Low-density lipoprotein receptor; LRP: LDLR-related protein; NTCP: Na^+ taurocholate co-transporting peptide; OATP: Organic anion transport polypeptides; SR-BI: Scavenger receptor class B type Ⅰ; (V)LDL: (Very) low-density lipoprotein.

HOW DOES CHOLESTEROL ORIGINATING FROM LIPOPROTEINS OR INTRACELLULAR SYNTHESIS REACH THE CANALICULAR MEMBRANE?

The hepatocyte is a polarized cell with a basolateral (sinusoidal) and an apical (canalicular) plasma membrane. Uptake of cholesterol from the plasma compartment occurs on the basolateral site (Figure 1), while biliary cholesterol secretion is an apical process (Figure 2). Hepatic cholesterol synthesis is carried out intracellularly. This polarization implies that a means of transport must exist for exogenous as well as endogenously synthesized cholesterol to reach the site where the biliary secretion process takes place.

Of note, cholesterol is also used for the synthesis of bile acids, and a number of factors modulating bile acid synthesis impact on hepatic cholesterol homeostasis. However, the regulation of bile acid synthesis will not be discussed, we refer to recent comprehensive reviews covering this topic[23,24].

The detailed mechanisms of intracellular cholesterol trafficking in hepatocytes are not understood thus far, especially how these relate to biliary cholesterol secretion. However, a few known transport proteins have been studied in more detail to test the effect of gain- or loss-of-function manipulations on biliary cholesterol secretion.

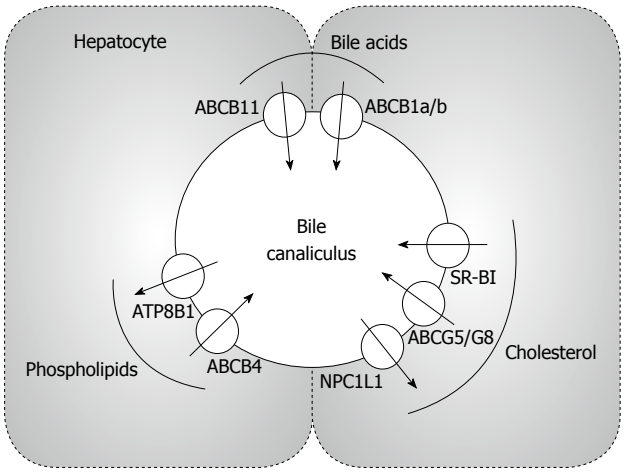

Figure 2 Overview of canalicular transporters/proteins that are involved in biliary bile acid, cholesterol and phospholipid secretion. ABC: ATP-binding cassette transporter; ATP8B1: ATPase class Ⅰ type 8B member 1; SR-BI: Scavenger receptor class B type Ⅰ; NPC1L1: Niemann-Pick C1-like protein 1.

A prominent example of such a protein is the Niemann-Pick type C protein 1 (NPC1) that plays a role in the intracellular trafficking of lipoprotein-derived cholesterol[25,26]. Specifically, NPC1 is involved in moving free cholesterol from the late endosomal/lysosomal compartment to the cytosol[25,26]. On a chow diet, mice lacking NPC1 expression had 37% higher biliary cholesterol secretion rates compared with controls[27]. However, when fed a 2% cholesterol-containing diet, biliary cholesterol secretion was significantly decreased in NPC1 knockout mice, while it increased 3.7-fold in wild-type controls[27]. These results indicated that NPC1 may become critical under conditions of high cholesterol intake. In turn, hepatic overexpression of NPC1 increased biliary cholesterol secretion by approximately 2-fold in chow-fed wild-type and high cholesterol diet-fed NPC1 knockout mice. However, no effect was observed in wild-type mice fed a 2% cholesterol diet[27]. The interpretation of this latter result is not straightforward, but other cholesterol metabolism pathways are also affected by NPC1, and changes in hepatic cholesterol content may play an additional role in explaining the observed phenotypes. Although more work is required to provide a detailed understanding of the role of NPC1, the presently available studies indicate that NPC1 is important in regulating the availability of cholesterol at the canalicular membrane for the biliary secretion process. Also NPC2, which plays a very similar role in intracellular cholesterol trafficking[28], is expressed in liver, is detectable in bile, and may be involved in the transport of cholesterol destined for biliary secretion. However, thus far no mechanistic studies have been performed, but a significant increase in hepatic NPC2 expression in gallstone-susceptible *vs* gallstone-resistant mouse strains has been reported[29]. Also the expression of another carrier protein, the sterol carrier protein-2 (SCP2), impacts biliary cholesterol secretion, with SCP2 overexpression increasing biliary secretion of cholesterol[30,31] and decreased SCP2 expression having the opposite effect[32]. Members of the steroidogenic acute regulatory (StAR) protein family of cholesterol transport molecules may also represent candidates to influence biliary cholesterol secretion[33,34]. However, thus far it has only been shown that StARD1 overexpression increases bile acid synthesis[35], while the absence of StARD3 in knockout mice had no impact on biliary sterol secretion[36]. In addition to intracellular cholesterol transport proteins, enzymes modulating the amount of free cholesterol present within a hepatocyte could also be expected to impact biliary cholesterol secretion. While this was shown to be the case for the neutral cholesteryl ester hydrolase[37], decreasing ACAT2 expression had no effect on biliary cholesterol output[38].

WHICH TRANSPORTERS ARE INVOLVED IN BILIARY CHOLESTEROL SECRETION?

On the apical membrane several transporters are directly involved in the biliary cholesterol secretion process. Transporter expression and activity is regulated by transcriptional as well as posttranscriptional mechanisms. Important transcriptional regulators are the nuclear hormone receptors liver X receptor (LXR; 2 isoforms LXRα, NR1H3, and LXRβ, NR1H2) and farnesoid X receptor (FXR, NR1H4), among others. For the specifics of LXR- and FXR-mediated gene regulation we refer to recent articles dealing with these topics[23,39-41]. In general, LXR is a nuclear receptor activated by oxysterols and functions as a sterol sensor exerting control on cholesterol metabolism[39,40]. As a general scheme, LXR activation stimulates metabolic processes favoring cholesterol elimination from the body, e.g. by increasing biliary cholesterol secretion *via* increased expression of ABCG5/G8[42] (see below) or by increasing the conversion of cholesterol to bile acids *via* increased expression of the cholesterol 7α-hydroxylase gene, at least in rodents[43].

On the other hand, FXR is the nuclear receptor activated by bile acids and orchestrates an adaptive response of the hepatocyte to altered bile acid levels[23,41]. In cholestatic conditions, FXR induces a downregulation of bile acid synthesis[23], a reduction in bile acid uptake into hepatocytes by decreasing the expression of the cellular uptake receptors[23] and an increased biliary secretion of bile acids by increasing the expression of specific transporters such as ABCB11[44] (see below).

In general, biliary bile acid secretion is the main driving force for the secretion of phospholipids and cholesterol[45]. Infusion of bile acids results in a dose-dependent increase in biliary cholesterol secretion[46]. However, cholesterol secretion into the bile also critically depends on functional biliary phospholipid secretion which is required for the formation of mixed micelles[46]. This is evidenced by the Abcb4 knockout mouse, that in the absence of this key biliary phospholipid transporter almost completely lacks biliary cholesterol secretion[47,48]. The following transporters are specifically involved in these processes:

ABCB4

ABCB4 has historically been named multi-drug resistance P-glycoprotein 2 (MDR2). ABCB4 functions as a phosphatidylcholine (PC) flippase translocating PC from the inner to the outer leaflet of the canalicular membrane[45]. Biliary cholesterol secretion is fully dependent on the functionality of ABCB4 and thereby biliary phospholipid secretion. In Abcb4-deficient animals, biliary cholesterol secretion is virtually absent[47,48]. Furthermore, PC decreases the toxic effects of bile acids on the canalicular membrane, and Abcb4 knockout mice as well as patients lacking this transporter develop a progressive liver disease[49,50].

ATPase class I type 8B member 1

ATPase class I type 8B member 1 (ATP8B1) is a P-type ATPase that flips phosphatidylserine (PS) from the outer to the inner leaflet of the canalicular membrane resulting in a reduction in the PS content and a consecutive increase in the sphingomyelin content of the outer canalicular leaflet[51,52]. In humans, mutations in *ATP8B1* cause severe chronic or periodic cholestatic liver disease[53,54]. A mutation resulting in severe liver disease is the glycine to valine substitution at amino acid 308 (G308V)[54]. In mice carrying the $Atp8b1^{G308V/G308V}$ mutation Atp8b1 is almost completely absent, which causes enhanced biliary excretion of PS and also cholesterol, mainly due to decreased rigidity of the outer canalicular leaflet (see below)[52,55].

ABCB11

ABCB11 is classically referred to as the bile salt export pump (BSEP; also known as sister of P-glycoprotein) and is mediating biliary secretion of bile acids[44,56]. A 2-fold increase in ABCB11 expression in transgenic mice resulted in increased biliary output of bile acids[57]. Notably, expression of other biliary transporters remained unaltered providing additional evidence that biliary bile acid secretion is the driving force for the secretion of the other lipid species into bile[57]. However, in Abcb11 knockout mice, there is still substantial residual biliary bile acid secretion and the expression of Mdr1a (Abcb1a) and Mdr1b (Abcb1b) was found to be upregulated indicating a potential compensation mechanism[58]. Subsequently, triple knockout mice lacking all 3 transporters have been generated to prove this concept, and indeed these mice develop an extreme cholestatic phenotype[59].

How do these transporters work together in the process of bile formation? First, the specific properties of the canalicular membrane need to be considered, since this has to fulfill 2 key functions: (1) withstand extremely high concentrations of bile acids, that are powerful detergents able to basically solubilize normal plasma membranes resulting in cell death; and (2) still allow for regulated secretion of the different bile components. The answer to these questions seems to lie in the asymmetry of the canalicular membrane with a high content of sphingomyelin and cholesterol in the outer leaflet[60]. In membranes composed of glycerophospholipids (PC, PS and phosphatidylethanolamine) lipids are loosely packed, the so-called "liquid disordered" phase[61,62]. Addition of sphingomyelin and cholesterol induces a more rigid membrane structure, the so-called "liquid ordered" phase that does not allow for the intercalation of detergents and thereby the membrane is rendered increasingly resistant against detergents[61,63,64]. The task of ATP8B1 in this model would be to preserve the rigid structure of the outer canalicular leaflet by inward flipping of PS. The amphipathic bile acids are actively excreted into the canalicular lumen by ABCB11, where they form simple micelles in the aqueous environment of the bile[9,46]. Phospholipids are translocated to the outer leaflet of the canalicular membrane by ABCB4 where they are added to the simple micelles resulting in mixed micelles[9,46]. However, the precise mechanism of how this occurs is still elusive. Subsequently, cholesterol is then taken up into these mixed micelles[9,46]. The authentic cholesterol transporters that play a role in this process are discussed below.

ABCG5/G8

The transport system contributing quantitatively the major amount of cholesterol secretion into the bile is the obligate heterodimer transporter pair ABCG5/ABCG8[65-67]. These are expressed almost exclusively in the liver and the intestine, and respective mutations have been identified as the disease substrate for sitosterolemia, a rare autosomal recessive disorder which is characterized by the accumulation of plant sterols in blood and tissues[68,69]. This accumulation is caused by increased sterol absorption from the diet and decreased biliary sterol secretion[70,71]. In the intestine, ABCG5/G8 secrete absorbed plant sterols back into the intestinal lumen, while on the bile canaliculus ABCG5/G8 mediate biliary plant sterol as well as cholesterol secretion into bile[70,71]. Since in healthy individuals, plasma plant sterol levels are very low, ABCG5/G8 represent, under physiological conditions, mainly a transport system for cholesterol. This is mirrored by the fact that biliary cholesterol secretion in ABCG5 and/or ABCG8 knockout mice is reduced by 75%[71-73]. In turn, transgenic overexpression of ABCG5/G8 resulted in an increase in biliary cholesterol secretion that was directly proportional to the copy numbers of the transgene over a wide range, indicating that under these conditions neither delivery of cholesterol to the transporters nor the level of cholesterol acceptors within the bile are rate-limiting[74]. In addition, ABCG5 and ABCG8 are targets of the nuclear hormone receptor LXRα and the increase in biliary cholesterol secretion observed upon LXR activation with endogenous or synthetic ligands depends largely on functional ABCG5/G8 expression[75]. Interestingly, also the increasing effects of cholate as well as diosgenin on biliary cholesterol secretion depend on the functional expression of ABCG5/G8 and subsequently are not seen in mice lacking either or both of the transporters[74,76]. Notably, there are as yet ill-defined ABCG5/G8-independent pathways of biliary cholesterol secretion[77] (see also below).

With relevance to atherosclerotic CVD, when human ABCG5/G8 transgenic mice overexpressing the transgene

in the liver as well as in the intestine were crossed into the atherosclerotic LDLR$^{-/-}$ genetic background, these mice developed significantly less atherosclerosis compared with wild-type controls[78]. On the other hand, only hepatic overexpression of ABCG5/G8 does not alter atherosclerosis in LDLR$^{-/-}$ as well as apoE$^{-/-}$ mice[79], unless intestinal cholesterol absorption is also reduced by ezetimibe[80]. With relevance to gallstone disease, recently specific mutations in ABCG5/G8, namely ABCG5 R50C and ABCG8 D19H, have been identified in humans and shown to increase the risk for cholesterol gallstone disease[81-83]. Since the role of ABCG5/G8 is to increase biliary cholesterol secretion, and cholesterol gallstone formation requires increased amounts of cholesterol within bile, these respective mutations are likely to constitute a gain-of-function phenotype. However, since these association data were obtained in human studies, this concept still requires verification in an experimental setting that allows determination of cause-effect relationships.

Although several studies have shown that the ABCG5/G8 heterodimer is a key component of biliary cholesterol secretion[70,71], the molecular mechanism by which this transporter pair mediates biliary cholesterol secretion at the canalicular membrane has not been yet elucidated. This lack of insight is mainly due to the unavailability of easy to study model systems such as polarized hepatocyte cell lines, or simple and reliable methods to isolate and characterize pure apical and basolateral membranes. However, the currently most accepted model suggests that ABCG5/ABCG8 act as a liftase elevating cholesterol just sufficiently outside of the outer leaflet of the canalicular membrane to be extracted by mixed micelles[84].

Niemann-Pick C1-like protein 1

The Niemann-Pick C1-like protein 1 (NPC1L1) was originally identified as a key regulator of intestinal cholesterol absorption and the molecular target of the cholesterol absorption inhibitor ezetimibe[85,86]. It is also highly expressed in human liver, but not in rodents[87]. In human hepatocytes, NPC1L1 is localized to the canalicular membrane[88]. Similar to its role in the intestine, hepatic NPC1L1 apparently facilitates the uptake of newly secreted biliary cholesterol[89]. In NPC1L1 transgenic mice with hepatic overexpression of the transgene, a more than 90% decrease in biliary cholesterol concentration is observed without any effect on biliary phospholipid and bile acid levels. Interestingly, treatment with ezetimibe normalizes biliary cholesterol concentrations in hNPC1L1 transgenic animals[89]. Therefore, particularly in humans, ezetimibe supposedly reduces plasma cholesterol levels by inhibiting both intestinal as well as hepatic NPC1L1 activity. However, the regulation of this important cholesterol transporter and modifier of hepatic cholesterol secretion is still incompletely understood.

SR-BI

SR-BI has been characterized as a receptor for HDL cholesterol, mediating bi-directional cholesterol flux, either efflux or selective uptake, depending on concentration gradients[90,91]. In contrast to ABC transporters, SR-BI apparently requires no energy consumption[91]. SR-BI is mainly expressed in the liver and in steroidogenic tissue[90,91]. In hepatocytes, SR-BI mediates the internalization of HDL cholesterol without the concomitant catabolism of HDL apolipoproteins[90,91]. SR-BI is detectable in hepatocytes *in vivo* at both the basolateral as well as the apical membrane[92,93]. Since HDL-derived cholesterol is a major source of sterols designated for biliary secretion[15], these properties of SR-BI suggest a potential involvement in biliary cholesterol secretion.

Cholesterol uptake from HDL *via* SR-BI can be increased in 2 different ways, by modulating the properties of the ligand, namely HDL, as well as by changing the expression level of SR-BI. HDL modification by phospholipases decreases its phospholipid content, destabilizes the particle and makes the HDL cholesterol ester more susceptible towards SR-BI-mediated selective uptake[94-97]. Overexpression of 2 phospholipases relevant for human physiology in mice, namely group IIA secretory phospholipase A2 (sPLA2)[98,99] and endothelial lipase (EL)[95,97,100] has 2 major metabolic effects: (1) in plasma, HDL cholesterol levels decrease significantly, and (2) hepatic cholesterol levels increase[94,101]. While SR-BI expression or membrane localization[94,101] was not affected, consecutive *in vitro* studies showed that SR-BI-mediated selective uptake from sPLA2- or EL-modified HDL was increased by 77%[94] and 129%[97], respectively, explaining the increased hepatic cholesterol uptake rates. However, despite this increased SR-BI-mediated hepatic cholesterol uptake, biliary cholesterol secretion in sPLA2 transgenic mice or mice with adenovirus-mediated EL overexpression was essentially unchanged compared with controls[94,101]. As a third example, the absence of hepatic lipase also did not affect biliary cholesterol secretion, although in this study actual SR-BI-mediated uptake of HDL cholesterol was not quantified[102]. Overall, these data demonstrate that biliary cholesterol secretion remains unaltered when only the ligand, HDL, but not SR-BI expression itself is modulated.

In contrast, changes in the hepatic expression level of SR-BI directly translate into altered biliary cholesterol secretion rates. SR-BI knockout mice have biliary cholesterol secretion rates reduced by 55%[103]. Consistent with these data, adenovirus-mediated SR-BI overexpression in the liver of wild-type mice increases gallbladder cholesterol content[92] as well as biliary cholesterol secretion rates[93]. Interestingly, with relevance to disease, hepatic SR-BI overexpression increases reverse cholesterol transport[104] and is protective against atherosclerotic CVD, even though plasma HDL cholesterol levels are decreased[105,106]. In gallstone-susceptible mice, hepatic SR-BI expression was higher and associated with biliary cholesterol hypersecretion[107]. In addition, hepatic mRNA as well as protein expression of SR-BI was higher in Chinese patients with cholesterol gallstone disease compared with controls and even correlated with an increased cholesterol saturation

index[108] suggesting that the link between SR-BI and biliary cholesterol secretion might be relevant for human pathophysiology as well. In this study, increased expression of LXRα was also noted in the livers of gallstone patients[108], and previous experimental work suggested that modulation of SR-BI expression results in altered expression of LXR target genes[109].

However, increased biliary cholesterol secretion in response to SR-BI overexpression was completely independent of LXR as evidenced by the use of knockout mice[93]. Surprisingly, this biological effect was also independent of functional expression of ABCG5/G8, since in ABCG5 knockout mice hepatic overexpression of SR-BI increased biliary cholesterol secretion rates to the levels of wild-type control mice and thereby normalized the cholesterol secretion deficit caused by absent ABCG5 expression[93]. To a certain extent canalicular SR-BI also acts independent of ABCB4, since hepatic overexpression of SR-BI in ABCB4 knockout mice resulted in significantly higher biliary cholesterol secretion, although in terms of mass secretion this effect was minor, indicating that cholesterol secreted *via* SR-BI still requires mixed micelles as acceptors[93]. Interestingly, SR-BI overexpression particularly augmented canalicular expression of the receptor[93]. Since, in SR-BI overexpression conditions, increased canalicular SR-BI localization was associated with an increased cholesterol content of the canalicular membrane and higher biliary cholesterol secretion rates[93], conceivably this biological effect was mediated by SR-BI. SR-BI requires a cholesterol gradient for transport[91], which is, however, constantly provided within the canaliculus by the direction of bile flow and the associated transport of cholesterol away from the hepatocyte. It would be interesting and physiologically relevant to explore whether SR-BI also contributes a significant part to the ABCG5/G8-independent biliary cholesterol secretion under steady-state conditions and not only has an effect in response to overexpression.

It is presently unclear how cholesterol secreted by SR-BI into the bile reaches the canalicular membrane. One possibility could be that SR-BI itself mediates this transport, since a transcytotic route of trafficking from the basolateral to the apical membrane has been proposed for SR-BI in a process that is dependent on microtubuli function[110]. However, even under conditions when microtubuli function and thereby transcytotic transport were efficiently abolished *in vivo*, SR-BI overexpression still significantly increased biliary cholesterol secretion[93]. These data indicate that microtubuli function is not required for the increasing effect of SR-BI on biliary cholesterol secretion. Other, as yet not characterized pathways, are therefore likely to contribute.

WHAT DIRECTIONS SHOULD FUTURE RESEARCH TAKE?

In our view, there are a number of interesting and relevant questions that are unresolved and should be addressed by future research in the field. The contribution of SR-BI to the ABCG5/G8-independent biliary cholesterol secretion under steady-state conditions would be important to know. Related to this, but also extending beyond SR-BI, more work appears to be required to identify and delineate the intracellular pathways for cholesterol transport that contribute to biliary cholesterol secretion. In addition to the selective uptake pathway for HDL cholesterol that is mediated by SR-BI, a holoparticle uptake pathway has also been characterized that is responsible for approximately a quarter of the total HDL cholesterol taken up into the liver[97,111-114]. However, whether this pathway contributes cholesterol for biliary secretion is currently unknown. Expanding this question, there also appears to be scope for assessing the differential contribution of apoB-containing lipoproteins *vs* HDL in bile acid synthesis and the respective biliary secretion of cholesterol and bile acids. Further research on these pathways would not only be relevant to increase our mechanistic insights into pathophysiology but also to define and characterize potential novel therapeutic targets for the treatment of atherosclerotic CVD and cholesterol gallstone disease.

CONCLUSION

Biliary cholesterol secretion is important for 2 major disease complexes, atherosclerotic CVD and cholesterol gallstone disease. Research thus far has provided valuable understanding of the regulation of biliary cholesterol secretion. With the identification of the ABC transporters mediating the biliary secretion of bile acids (ABCB11), phospholipids (ABCB4) and cholesterol (ABCG5/G8) the major transport proteins for the respective physiological processes have been delineated. However, more recently a number of proteins that modulate or mediate biliary cholesterol secretion such as the phosphatidylserine flippase ATP8B1, NPC1L1 and SR-BI have gained attention. Although several proteins are currently known that impact biliary cholesterol secretion, further research into the mechanisms determining their respective activities as well as the routes of intrahepatic cholesterol trafficking and the regulation of hepatic cholesterol pools is required.

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TOPIC HIGHLIGHT

Astrid van der Velde, PhD, *Series Editor*

A new framework for reverse cholesterol transport: Non-biliary contributions to reverse cholesterol transport

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Abstract

Reduction of low-density lipoprotein-cholesterol through statin therapy has only modestly decreased coronary heart disease (CHD)-associated mortality in developed countries, which has prompted the search for alternative therapeutic strategies for CHD. Major efforts are now focused on therapies that augment high-density lipoprotein (HDL)-mediated reverse cholesterol transport (RCT), and ultimately increase the fecal disposal of cholesterol. The process of RCT has long been thought to simply involve HDL-mediated delivery of peripheral cholesterol to the liver for biliary excretion out of the body. However, recent studies have revealed a novel pathway for RCT that does not rely on biliary secretion. This non-biliary pathway rather involves the direct excretion of cholesterol by the proximal small intestine. Compared to RCT therapies that augment biliary sterol loss, modulation of non-biliary fecal sterol loss through the intestine is a much more attractive therapeutic strategy, given that excessive biliary cholesterol secretion can promote gallstone formation. However, we are at an early stage in understanding the molecular mechanisms regulating the non-biliary pathway for RCT, and much additional work is required in order to effectively target this pathway for CHD prevention. The purpose of this review is to discuss our current understanding of biliary and non-biliary contributions to RCT with particular emphasis on the possibility of targeting the intestine as an inducible cholesterol secretory organ.

Key words: Cholesterol; Intestine; Bile; Lipoprotein; Reverse cholesterol transport

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INTRODUCTION

Atherosclerosis is the disease process underlying the clinical complications of coronary heart disease (CHD), an epidemic that has taken more lives in the United States over the past century than the next four causes combined[1]. Atherosclerotic cardiovascular disease (ASCVD) was long thought to be a problem of developed Western societies, but it is now understood that this cholesterol-driven disease pervades throughout most of the world[2,3]. The most accurate predictor of ASCVD incidence is the plasma concentration of low-density lipoprotein cholesterol (LDLc). Hence, lowering LDLc has been the primary therapeutic goal for decades, and statins have been the main drug used to accomplish this. However, even with the substantial

LDLc lowering achieved with statin treatment, these drugs have been able to reduce CHD-associated mortality and morbidity by only approximately 30%[4]. Major pharmaceutical interest has thus shifted towards developing high-density lipoprotein cholesterol (HDLc)-elevating agents, since HDLc was shown to be an even stronger predictor than LDLc for CHD in the Framingham Heart Study[5]. There have been many mechanisms proposed as to how HDLc influences the development of CHD[6-8], but the most widely accepted is the idea that HDL directly facilitates the process of reverse cholesterol transport (RCT).

REVERSE CHOLESTEROL TRANSPORT: THE CLASSIC "BILIARY" MODEL REQUIRES RECONSIDERATION

Nearly forty years ago, Glomset *et al*[9,10] presented the seminal framework of HDL-driven RCT. This model for RCT was described as a process by which extrahepatic (peripheral) cholesterol is returned to the liver for excretion into bile for subsequent loss through the feces[9,10]. It is reasonable to assume that the process of RCT has primarily evolved to protect multiple peripheral tissues from the excess accumulation of unesterified cholesterol, which is known to be cytotoxic[11,12]. Importantly, in the context of ASCVD, RCT is thought to involve HDL-mediated efflux of cholesterol from the arterial wall, specifically from cholesterol-laden macrophages[13-15]. In the classic "biliary" view of RCT[9,10], HDL-mediated delivery of peripheral cholesterol to the liver directly results in biliary secretion[13-15]. Given this model, plasma HDLc levels should accurately predict both biliary sterol secretion and fecal sterol loss. However, there are now several examples where plasma HDLc levels do not predict the levels of cholesterol in bile or feces[16-18]. For example, in mice lacking ATP-binding cassette protein A1 (ABCA1) or apolipoprotein-AI, plasma HDLc is essentially at undetectable levels[16-18]. However, in the face of near complete absence of HDLc, biliary and fecal cholesterol levels are normal in these mice[16-18]. These studies quite convincingly demonstrate that plasma HDLc levels do not determine the amount of cholesterol ultimately excreted into the feces. These findings bring into question whether therapeutic strategies that simply raise HDLc will indeed promote fecal disposal of cholesterol. Ultimately, to effectively target RCT, a drug should show efficacy in promoting the fecal disposal of cholesterol, not simply raising HDLc.

Another problem with the classic model of RCT is that the levels of cholesterol secreted into bile do not accurately predict fecal disposal of cholesterol[19-24]. In support of this concept, it has been demonstrated in several mouse models where hepatobiliary cholesterol secretion is substantially diminished that fecal sterol loss is normal[19-23]. The first demonstration of this was seen in mice lacking the canalicular phospholipid transporter, multidrug resistance 2 (Mdr2)[19]. Mice lacking Mdr2 have the inability to secrete phospholipids into bile, and secondary to biliary phospholipid insufficiency these mice also have diminished biliary cholesterol secretion[19]. Quite strikingly, even with > 80% reduced levels of biliary cholesterol, Mdr2 null mice have normal or even increased fecal cholesterol loss compared to wild type mice[19,20]. Furthermore, activation of the liver X receptor (LXR) in Mdr2 null mice results in large increases in fecal sterol output, without increasing biliary cholesterol output, supporting the presence of an LXR-inducible non-biliary pathway for fecal sterol loss in these mice[20]. Our group has found strikingly similar results in another mouse model lacking the ability to secrete cholesterol into bile[21,22]. In order to characterize the role of Niemann-Pick C1-Like 1 (NPC1L1) in hepatobiliary sterol secretion, we previously generated mice transgenically overexpressing NPC1L1 in hepatocytes[21]. Interestingly, NPC1L1 overexpression in the liver results in a > 90% reduction in biliary cholesterol levels, yet mass fecal neutral sterol loss and macrophage RCT is normal in these mice[21,22]. In addition, LXR activation in NPC1L1-^{LiverTg} mice results in large increases in macrophage RCT and mass fecal neutral sterol loss[22], further supporting the existence of a non-biliary pathway in this model. In order to more definitively test whether bile is required for macrophage RCT, we set out to create a surgical model where there would be no biliary emptying into the small intestine, without obstructing bile flow. In order to do this we surgically diverted bile flow away from the small intestine and measured macrophage RCT. Importantly, mice surgically lacking biliary contributions to the intestine have normal LXR-inducible macrophage RCT[22]. Collectively, these studies in mice demonstrate that mass fecal sterol loss and macrophage RCT can proceed in the complete absence of biliary sterol secretion, challenging the obligate role of bile in RCT. Although the classic "biliary" model has been instrumental in providing a framework for our current understanding of RCT, we now understand that fecal sterol loss does not absolutely require circulating HDL[16-18] or biliary sterol secretion[19-22]. Paradigm shifting data such as these[16-23] strongly suggest that an alternative model for RCT is now appropriate. Based on our current understanding, fecal sterols originate from at least two distinct excretory routes: (1) the classic hepatobiliary sterol secretion route; and (2) the non-biliary liver to plasma lipoprotein to small intestine to feces route (Figure 1). Importantly, the major mechanisms regulating cholesterol secretion into bile have been recently defined[19,21,24-27], but almost no information exists regarding mediators of non-biliary cholesterol secretion by the intestine.

REVERSE CHOLESTEROL TRANSPORT: EARLY SUPPORT FOR AN ALTERNATIVE "NON-BILIARY" MODEL

Long before Glomset *et al*[9,10] proposed the "biliary" model for RCT, the existence of a "non-biliary" pathway for RCT was proposed by Sperry *et al*[28]. In 1927, Sperry's experiments demonstrated that in dogs that had undergone chronic biliary fistula surgery, fecal neutral sterol

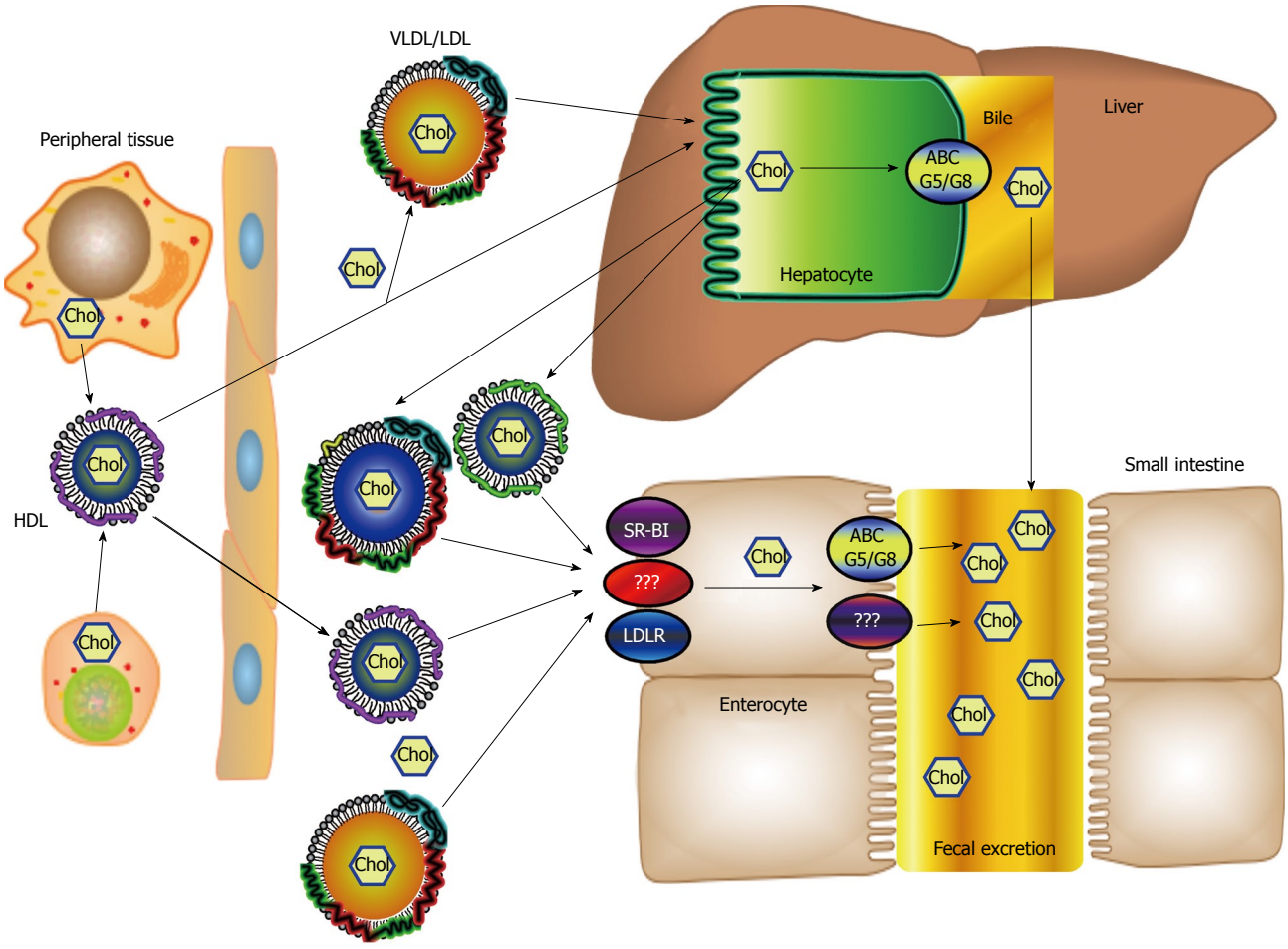

Figure 1 Biliary and non-biliary fecal cholesterol excretion. In this proposed model, cholesterol is effluxed from peripheral cells onto high-density lipoprotein (HDL) and then delivered to the liver or small intestine *via* HDL or very low density lipoprotein (VLDL)/low-density lipoprotein (LDL) following cholesterol ester transfer protein-mediated transfer. The cholesterol internalized or *de novo* synthesized by the liver is secreted into bile through the action of ABCG5/G8 and subsequently excreted in the feces. Alternatively, the liver secretes the cholesterol in apoB-containing lipoproteins or HDL containing apoAI or apoE, which are specifically targeted to the small intestine. After being internalized by scavenger receptor, class B, type I (SR-BI), LDL receptor (LDLR), or another lipoprotein receptor system, the cholesterol is trafficked across the enterocytes to the brush border membrane and effluxed into the intestinal lumen by ABCG5/G8 or a currently unidentified cholesterol transporter.

loss was paradoxically increased, compared to control bile intact dogs. Sperry's findings were largely ignored given that it was already well accepted in the 1920s that bile was the only source of fecal lipids. Nearly a half-century later, Pertsemlidis *et al*[29] confirmed the results of Sperry *et al*[28], by demonstrating surgical biliary diversion resulted in complete loss of fecal acidic sterol output, yet fecal neutral sterol output actually increased approximately 7-fold. These studies, along with elegant work done by Dietschy *et al*[30,31], have demonstrated that bile is absolutely required for the enterohepatic circulation of acidic sterols, but this is not the case for neutral sterols. Similar results have been seen in bile diverted rats[32] and familial hypercholesterolemic humans[33]. Actually, as early as 1959, it was suggested that non-dietary fecal cholesterol loss in humans consists of two distinct fractions: (1) the traditional fraction coming from hepatobiliary secretion; and (2) an elusive fraction directly secreted by the intestine[34]. Unfortunately, a major drawback of these early biliary diversion or obstruction models is the interruption of enterohepatic circulation of bile acids and other non-lipid components that are found in bile. Under bile-diverted conditions, the absence of bile salt delivery to the intestinal lumen compromises intestinal cholesterol absorption, and endogenous cholesterol synthesis is strongly upregulated locally in the intestine[30]. Given these limitations, these early studies have been largely ignored, and the theory that fecal neutral sterol loss derives only from a biliary origin has become dogma. However, we have recently demonstrated that biliary cholesterol secretion is not required for macrophage RCT in mice using both surgical and genetic models of biliary insufficiency[22]. Collectively, findings in animal models of bile diversion have clearly demonstrated the presence of a non-biliary component of RCT, yet the mechanisms underlying such a process are poorly understood.

RELATIVE CONTRIBUTION OF THE NON-BILIARY PATHWAY TO RCT

In addition to the classic RCT pathway involving choles-

terol secretion into the bile, there is mounting evidence that a non-biliary pathway quantitatively contributes to fecal cholesterol excretion. The non-biliary RCT pathway appears to involve the targeting of plasma cholesterol to the small intestine and the subsequent cellular secretion of the cholesterol into the lumen of this organ. Christened transintestinal cholesterol efflux (TICE) by Kuipers, Groen and their colleagues, the relative contribution of the non-biliary pathway to fecal cholesterol loss has not been fully defined. In normal chow-fed C57BL/6J mice, TICE accounts for 33% of total fecal neutral sterol loss[35], and roughly 20% of total fecal sterol loss in FVB mice[20]. However, it is important to point out that non-biliary fecal cholesterol loss is quite sensitive to pharmacological manipulation. To this end, LXR activation can dramatically augment non-biliary macrophage RCT[22], and increases mass fecal neutral sterol loss[20,22,35]. In fact, LXR activation in C57BL/6J increases the contribution of TICE to fecal cholesterol loss from 33% in vehicle treated mice to 63% in LXR agonist-treated mice[35]. Furthermore, activation of the peroxisome proliferator activated receptor δ (PPAR-δ) also promotes non-biliary neutral fecal sterol loss in mice[36]. Importantly, now in three independent genetically modified mouse models (ABCG5/G8$^{-/-}$, Mdr2$^{-/-}$, and NPC1L1-^{LiverTg}) that have severely diminished biliary cholesterol secretion, fecal neutral sterol loss is only modestly decreased[24] or not altered at all[20,22]. This clearly indicates that the non-biliary pathway must be able to adequately compensate for biliary insufficiency to maintain normal fecal cholesterol loss in rodents. Collectively, these data support the idea that fecal neutral sterol loss is a mixture of dietary, biliary, and intestinally-derived cholesterol, and the origins of the latter source likely originates from the plasma compartment[20,23,35]. Given the plasma source of intestinally derived fecal neutral sterols, and the central role of the liver in lipoprotein metabolism, it is tempting to speculate that the liver may serve as a potential site of organization for non-biliary fecal cholesterol loss.

ORIGINS OF NEUTRAL STEROLS FOR TRANSINTESTINAL CHOLESTEROL EFFLUX

In the classic view of RCT, the liver plays a central role in moving peripheral cholesterol out of the body[13-15]. In parallel, we believe that the liver also plays a central role in the non-biliary pathway by re-packaging peripheral cholesterol into liver-derived lipoproteins that are targeted for subsequent intestinal uptake[23]. In support of this concept, most of the mouse models where non-biliary fecal cholesterol loss has been described[20-23] represent conditions where free cholesterol could potentially accumulate in the liver due to defects in normal hepatic elimination pathways. It remains possible that under conditions where hepatic free cholesterol burden becomes too excessive for disposal through biliary secretion (i.e. Mdr2$^{-/-}$ mice[20], ACAT2 ASO treatment[22], or NPC1L1-$^{Liver\text{-}Tg}$ mice[22,23]), an alternative non-biliary plasma lipoprotein-based route for fecal cholesterol disposal is utilized. In previous work, we were able to show that the liver can secrete lipoprotein particles that preferentially deliver cholesterol to the proximal small intestine for fecal excretion[22]. However, whether these liver-derived lipoproteins represent nascent very low density lipoprotein (VLDL) particles, nascent HDL particles, or some novel lipoprotein remains to be addressed.

POSSIBLE COMPONENTS IN THE TRANSINTESTINAL CHOLESTEROL EFFLUX PATHWAY

Based upon their ability to transport mass amounts of cholesterol in the blood and deliver the cholesterol to specific cells within tissues and organs, lipoproteins are almost certainly involved in the TICE pathway. However, it is unclear which lipoprotein class or classes is involved and how the lipoprotein is targeted to the small intestine. Because of its role in the removal of excess cholesterol from peripheral tissues, HDL is a logical choice for the lipoprotein responsible for cholesterol trafficking to the small intestine. However, ABCA1 null mice, which have very low levels of HDL in plasma, have normal or increased fecal cholesterol excretion under basal conditions[16,18,37]. Furthermore, treatment with an LXR agonist, which stimulates TICE, causes fecal cholesterol excretion to increase to the same extent in both wild type and ABCA1 null mice[38]. Thus, at least in mice, HDL does not appear to be necessary for non-biliary RCT.

Apolipoprotein E (apoE)-rich HDL (HDL-E) could also be involved in delivering cholesterol to the small intestine for fecal excretion. HDL-E is found in the plasma of wild type mice[21] but its concentration is significantly increased in two mouse models with upregulated TICE, NPC1L1-^{LiverTg} and LXR agonist-treated wild type mice[21,39,40]. Compared to LDL and apoAI-rich HDL, HDL-E has an intermediate size and a higher ratio of free cholesterol to cholesteryl ester. The source of HDL-E is currently unknown, but it could be generated by hepatic ABCA1 effluxing cholesterol and phospholipid to lipid-poor apoE. Hepatic ABCA1 is increased in both NPC1L1-^{LiverTg} and LXR-agonist treated mice and it has been shown that hepatic overexpression of ABCA1 in mice increases the plasma concentration of HDL-E[41]. Humans and other species with high levels of cholesterol ester transfer protein activity have low concentrations of plasma HDL-E[42]. Thus, the contribution of HDL-E to the TICE pathway would presumably be much greater in mice than humans.

Evidence from our group also suggests that liver-derived apoB-containing lipoproteins can deliver cholesterol for TICE[23]. In order to study the hepatic function of the cholesterol esterifying enzyme ACAT2, we treated mice with an antisense oligonucleotide (ASO) which targets ACAT2 mRNA for degradation. Mice treated with ACAT2 ASO *vs* control ASO had no change in biliary cholesterol secretion but had a 2-fold increase in fecal cholesterol excretion. The increased fecal cholesterol excretion was concluded to be the result of TICE since

ACAT2 ASO-treated mice, unlike ACAT2 null mice, did not have a significant reduction in cholesterol absorption. To determine whether the liver was producing a lipoprotein that was targeted for clearance by the small intestine, isolated liver perfusion was conducted on mice that had been radiolabeled with [^{3}H]cholesterol and treated with control or ACAT2 ASO. The radiolabeled perfusate, which carried almost 100% of the cholesterol on VLDL, was then injected into control and ACAT2 ASO treated mice. After 6 h, 2-3 fold more [^{3}H]cholesterol from the ACAT2 ASO perfusate compared to the control ASO perfusate had accumulated in the lumen and wall of the proximal small intestine. One interpretation of this result was that the VLDL secreted from the ACAT2 ASO liver was preferentially targeted to the small intestine. It is also possible that following clearance of the perfusate VLDL by the liver, the [^{3}H]cholesterol was packaged into another lipoprotein that delivered the cholesterol to the intestine. However, hepatic production of this lipoprotein would likely be driven by some factor secreted exclusively into the ACAT2 ASO perfusate since intestinal accumulation of [^{3}H]cholesterol from ACAT2 ASO perfusate was similar in control and ACAT2 ASO-treated recipient mice.

Regardless of the lipoprotein class that delivers cholesterol to the small intestine, there must be receptors involved in the internalization or uptake of the lipoprotein cholesterol by the enterocytes. Scavenger receptor, class B, type I (SR-BI), which is expressed on the basolateral surface of enterocytes, is one receptor that could potentially play a role in TICE. SR-BI can bind VLDL, LDL, and HDL and subsequently mediates the selective uptake of the cholesteryl ester[43]. Intestinal expression of SR-BI also increases with LXR agonist treatment of mice[20,38]. However, when measured using an intestinal perfusion system, TICE was found to be significantly increased in SR-BI null mice[44]. Studies involving mice with intestine specific overexpression or deletion of SR-BI may aid in determining whether SR-BI plays a part in TICE.

If apoE-rich HDL or apoB-containing lipoproteins are involved in the TICE pathway then a member of the LDL receptor family may mediate the uptake of these particles by the enterocytes. The LDL receptor is expressed in the small intestine; however its expression at the protein level is downregulated in mice treated with LXR agonist[45]. Moreover, TICE appears to be stimulated in LDL receptor null mice treated with ACAT2 ASO compared to control ASO[23]. This data indicates that the LDL receptor is not necessary for TICE but does not exclude the possibility that other members of the LDL receptor family may compensate when the LDL receptor is absent.

Following delivery of lipoprotein cholesterol to the enterocytes, the ATP-binding cassette (ABC) transporters G5 and G8 appear to participate in pumping the cholesterol into the lumen of the small intestine. Expressed in the liver and small intestine[46], ABCG5/G8 functions as an obligate heterodimer and facilitates cholesterol excretion from the body by effluxing cholesterol from the apical surface of hepatocytes and enterocytes into bile and intestinal contents, respectively[24,25,46,47]. By employing stable isotope tracers, it was determined that the contribution of TICE to fecal cholesterol excretion decreased from 25% in wild type mice to 15% in ABCG5 null mice[35]. In addition, LXR agonist treatment of ABCG5/G8 null mice, unlike wild type mice, did not increase mass fecal neutral sterol loss[48] and macrophage reverse cholesterol transport[49]. Although this data indicates that ABCG5/8 plays a role in TICE, there appears to be multiple or redundant pathways for effluxing cholesterol from enterocytes into the intestinal lumen since TICE continues to function, albeit at lower efficiency, in the absence of ABCG5/G8.

METHODOLOGIES FOR MEASURING TRANSINTESTINAL CHOLESTEROL EFFLUX

Several different methodologies have been used to measure TICE or non-biliary excretion in mice. Taking into consideration dietary intake, biliary secretion, fractional absorption, and fecal excretion of cholesterol, it was calculated that under basal conditions in wild type mice, TICE contributed 20%-50% of the cholesterol in feces[20,50]. Using similar measurements, approximately 40% of fecal cholesterol excretion was attributed to TICE in wild type mice treated with LXR agonist[20]. By determining the kinetics of stable isotopically labeled cholesterol that was delivered orally and intravenously plus assessing the fate of *de novo* synthesized cholesterol that was isotopically labeled, it was determined that 33% and 63% of fecal cholesterol excretion in mice treated with vehicle or LXR agonist was the result of TICE[35].

Cholesterol secretion into the lumen of the small intestine was also observed when the intestine of wild type mice was perfused with bile acid; phospholipid micelles[36,44,50]. The rate of cholesterol secretion was found to be highest in the proximal third of the small intestine[50], and was dependent upon the concentration of the phospholipid in the micelles[44]. Since [^{14}C]cholesterol that was intravenously injected was found in the perfusate, a portion of the cholesterol secreted into the lumen was derived from the plasma. Moreover, the plasma cholesterol secreted in the lumen must have trafficked through and mixed with the cholesterol pool of the enterocytes since the specific activity of the cholesterol in the perfusate was similar to that of the mucosa but was 10-fold less than that of the serum[50]. Although these studies showed that bile salt:phospholipid micelles could mediate the efflux of cholesterol from the small intestine, the question remains whether TICE would occur if the micelles consisted of physiological concentrations of not only bile salt and phospholipid but also cholesterol.

DOES TICE PLAY A MAJOR ROLE IN HUMAN FECAL CHOLESTEROL EXCRETION?

Since most of the studies of the TICE pathway have been

conducted in mice, the question remains whether TICE contributes significantly to fecal cholesterol excretion in humans. Using an intestinal perfusion system, it was estimated that approximately 44% of total fecal cholesterol output originated from non-biliary sources in humans[51]. Yet, it cannot be determined from this data whether TICE was the source of the non-biliary sterol. The current methodologies used to measure TICE in murine models are invasive and therefore could not be implemented in humans. However, the procedures used to measure TICE in mice could be applied to non-human primates, whose lipoprotein and cholesterol metabolism closely resembles that of man. If in non-human primates TICE can be shown to play a significant part in fecal cholesterol excretion and to be amenable to pharmaceutical manipulation then future studies in humans could be warranted. Although our understanding of non-biliary fecal sterol excretion is still in its infancy, continued research surrounding this pathway has strong potential to lead to novel therapies for the prevention and/or treatment of ASCVD.

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TOPIC HIGHLIGHT

Astrid van der Velde, PhD, *Series Editor*

From blood to gut: Direct secretion of cholesterol *via* transintestinal cholesterol efflux

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Abstract

The reverse cholesterol transport pathway (RCT) is the focus of many cholesterol-lowering therapies. By way of this pathway, excess cholesterol is collected from peripheral tissues and delivered back to the liver and gastrointestinal tract for excretion from the body. For a long time this removal *via* the hepatobiliary secretion was considered to be the sole route involved in the RCT. However, observations from early studies in animals and humans already pointed towards the possibility of another route. In the last few years it has become evident that a non-biliary cholesterol secretion pathway exists in which the intestine plays a central role. This transintestinal cholesterol efflux (TICE) pathway contributes significantly to the total fecal neutral sterol excretion. Moreover, recent studies have shown that TICE is also sensitive to stimulation. As a consequence, the direct role of cholesterol secretion from blood *via* TICE makes the intestine a suitable and approachable target for cholesterol removal from the body and possibly reduction of atherosclerosis. In this review, the discovery and recent findings contributing to understanding the mechanism of TICE will be discussed.

Key words: Cholesterol; Intestine; Transintestinal cholesterol efflux

INTRODUCTION

Since the introduction of statins about 25 years ago, therapeutic lowering of cholesterol levels has been proven successful in decreasing the incidence of cardiovascular diseases (CVDs)[1]. In fact, the reduction of low density lipoprotein (LDL) cholesterol levels in blood by the inhibition of cholesterol biosynthesis using these 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors is still the most potent cholesterol-lowering therapy to date[2]. However, while the reduction of LDL cholesterol levels in blood helps to prevent the development of atherosclerosis, the underlying process of CVD, it does not seem to be sufficient enough for the reversal of already existing atherosclerotic lesions[3,4]. With better understanding of cholesterol metabolism, new ideas for therapies to prevent CVD have emerged. One of the most prominent attempts in the last decade is stimulating reverse cholesterol transport (RCT)[5-7]. RCT describes the different stages of cholesterol transport, from efflux from peripheral tissues to fecal excretion[8]. Passage through liver and bile before subsequent entry into the intestinal lumen has long been considered to be the most important, if not the only, route in RCT. However recent findings have shown the existence of a cholesterol secretion pathway that is independent of this hepatobiliary tract[9,10].

REMOVAL OF CHOLESTEROL *VIA* ITS REVERSE TRANSPORT

Since the introduction of the term RCT in the early 1970s, our knowledge of this cholesterol removal pathway has advanced to a great extent[8]. In brief, the pathway starts with the efflux of cholesterol from peripheral cells, including from macrophages in the arterial wall, by cholesterol transporters adenosine triphosphate-binding cassette transporter protein (ABC) A1 and G1 (Figure 1)[6,7]. This cholesterol is transported *via* lipoproteins to the liver where it can be taken up by hepatocytes *via* specific lipoprotein receptors. The lipoprotein that mediates this transport is the high density lipoprotein (HDL). However, recent findings in transgenic mice also include a role for LDL *via* the activity of cholesteryl ester transfer protein[11]. After uptake at the basolateral side, the cholesterol can be transferred to the bile *via* secretion at the canalicular membrane of the hepatocyte. The cholesterol transport over the canalicular membrane is mainly facilitated by a heterodimer complex formed by ABCG5 and ABCG8[12]. Biliary cholesterol enters the intestinal lumen, from where a significant amount is re-absorbed by the enterocytes[13]. The remaining cholesterol leaves the body *via* fecal excretion.

This hepatobiliary pathway was considered to be the sole route for cholesterol secretion from the body. *In vivo* cholesterol balance studies in wild type and mouse models with abrogated biliary cholesterol secretion have, however, suggested the existence of an alternative direct cholesterol transport route from blood to the intestine.

TRANSINTESTINAL CHOLESTEROL TRANSPORT

If the fecal sterol output only depends on the hepatobiliary cholesterol transport, it should directly be affected by diminished biliary cholesterol secretion. Hence, fecal sterol excretion should be attenuated in mice lacking a functional ABCG5/ABCG8 or deficient for phospholipid transporter ABCG4. Interestingly, despite the very low to absent biliary cholesterol secretion in these mice their sterol output is similar to that of their wild type littermates[9,12,14]. The idea that the fecal sterol excretion might to a certain extent be independent of biliary cholesterol secretion is not confined to recent observations. Earlier findings going as far back as the beginning of the last century already suggested the existence of an additional source for cholesterol excretion. In 1927, Sperry[15] reported that he had measured more excreted cholesterol in the feces of dogs with bile fistula than he would have predicted based on the results obtained from control dogs; an observation which was confirmed 50 years later by Pertsemlidis *et al*[16]. It was also observed in rats that a large part of the cholesterol that ends up in the feces seems to originate from a source other than bile and diet[17,18].

To test the possibility that the intestine is not only responsible for the uptake of cholesterol but also is actively involved in the secretion of cholesterol, intestine perfusions were performed in mice[9]. In these experiments the bile duct was ligated and the bile itself was diverted *via* gallbladder cannulation. Subsequently, a section of the small intestine was perfused with a buffered solution containing a mixture of bile salts and phospholipids. This procedure allows a determination of the contribution of biliary cholesterol secretion, as well as that of transintestinal cholesterol secretion, to the total fecal neutral sterol output. This method demonstrated that a considerable amount of cholesterol is secreted by the small intestine. The secretion takes place over the entire length of the small intestine but it is most significant in the proximal part. The majority of this secreted cholesterol does not find its origin in the high turnover of enterocytes and does not correlate with cholesterol synthesis in the enterocytes. Moreover, intestine perfusions performed on mice that were intravenously injected with radiolabeled cholesterol established the direct secretion of cholesterol from blood through the intestinal wall into the intestinal lumen[9,19]. Surprisingly, these reports also showed that this transintestinal cholesterol efflux (TICE) pathway in mice plays a more prominent role in the excretion of cholesterol than the hepatobiliary route.

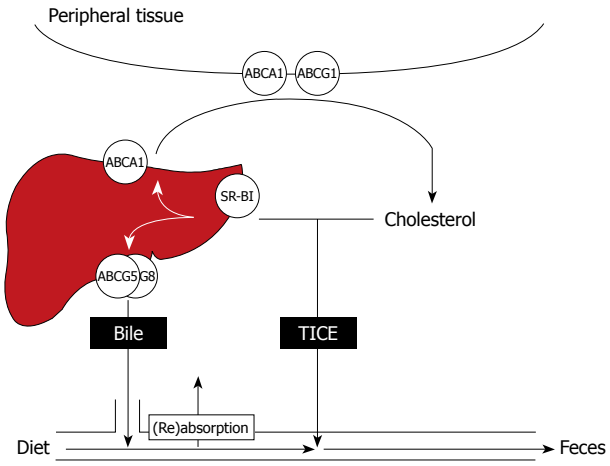

Figure 1 Schematic overview of the reverse cholesterol transport pathways. ABCA1: Adenosine triphosphate (ATP)-binding cassette transporter A1; ABCG1: ATP-binding cassette transporter G1; ABCG5: ATP-binding cassette transporter G5; ABCG8: ATP-binding cassette transporter G8; SR-BI: Scavenger receptor class B type I; TICE: Transintestinal cholesterol efflux.

While the intestine perfusions in mice showed for the first time the existence of TICE, a recent study by Brown *et al*[10] also unmistakingly showed the presence of a non-biliary route for cholesterol excretion. In their report, mice deficient for hepatic acyl-CoA: cholesterol O-acyltransferase 2 (ACAT2) had an increased fecal excretion of cholesterol without a changed biliary cholesterol secretion. Additionally, they also found intestinal cholesterol secretion to be the highest in the proximal small intestine.

With the evidence that the small intestine is not only involved in the uptake of cholesterol but also has the ability to actively secrete cholesterol, a new potentially attractive target for therapeutic lowering of cholesterol has emerged. However, more understanding of the mechanism of TICE is required.

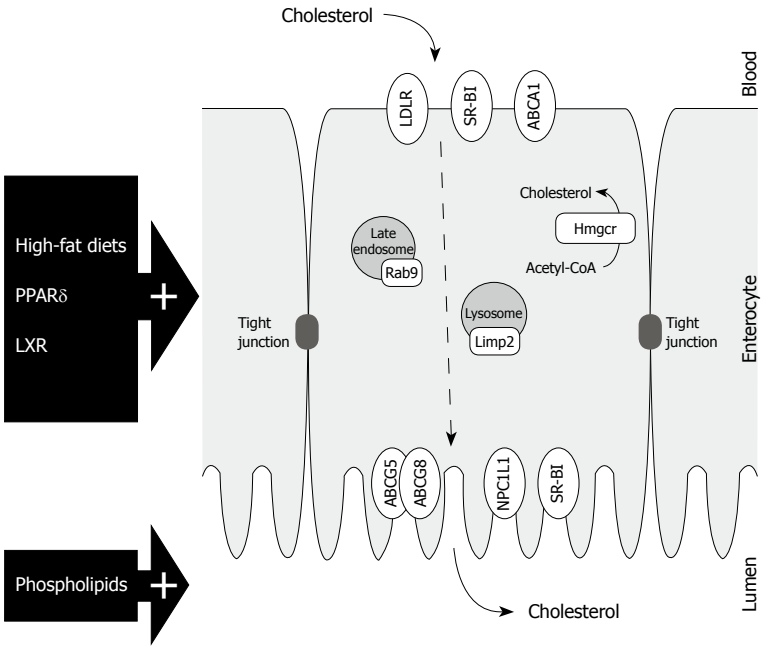

Figure 2 Enterocyte representation with a schematic overview of cholesterol transporters and potential transintestinal cholesterol efflux-related factors as discussed in this paper. PPARδ: Peroxisome proliferator-activated receptor δ; LXR: Liver X receptor; LDLR: Low density lipoprotein receptor; SR-BI: Scavenger receptor class B type I; ABCA1: Adenosine triphosphate (ATP)-binding cassette transporter A1; ABCG5: ATP-binding cassette transporter G5; ABCG8: ATP-binding cassette transporter G8; Hmgcr: 3-hydroxy-3-methyl-glutaryl-CoA reductase; Limp2: Lysosomal integral membrane protein 2; NPC1L1: Niemann-Pick C1-like 1 protein; TICE: Transintestinal cholesterol efflux.

IN VIVO STIMULATION OF THE TRANSINTESTINAL CHOLESTEROL EFFLUX PATHWAY

Several studies in mice have demonstrated that the activity of TICE can be stimulated. One of our earliest studies on TICE involved the sensitivity of this novel pathway to dietary manipulations. When mice are fed a high lipid diet, fecal neutral sterol excretion is increased[20-22]. Besides the higher input of cholesterol, this increase upon feeding with diets containing high cholesterol can be explained by reduced intestinal uptake of cholesterol and increased biliary cholesterol secretion. However, the increased fecal neutral sterol excretion seen in mice fed a high fat diet without cholesterol did not correlate with increased biliary cholesterol secretion. We studied the effect of high lipid diets on TICE and found that the secretion of cholesterol by the intestine is also increased in mice fed a western-type diet, containing high cholesterol and high fat[9]. Interestingly, a high cholesterol-only diet did not affect TICE suggesting that this secretion pathway is responsive to the fat content of the western-type diet[21]. This was confirmed in mice that were fed a high-fat diet without cholesterol. We also showed that activation of the peroxisome proliferator-activated receptor δ (PPARδ) caused an over two-fold increase in TICE[23]. This finding strengthened the link between TICE and fat metabolism since polyunsaturated fatty acids are natural ligands of this member of the nuclear receptor family[24].

Another nuclear receptor that is involved in the regulation of lipid-related metabolic processes, the liver X receptor (LXR), affects TICE as well[19,25]. In mice, activation of LXR increased fecal neutral sterol loss independent of biliary cholesterol secretion. However, the stimulation of TICE upon a high fat diet in LXRα knock-out mice suggests that LXR sensitivity of TICE occurs *via* a different pathway[22].

The effect of luminal modifications on TICE was investigated by means of introducing different combinations of bile salt-phospholipid[21]. Although the intraluminal presence of bile salts was crucial for TICE, an important finding was that, in contrast to biliary cholesterol secretion, TICE seemed to be insensitive to both the type of bile salt and bile salt concentration. The presence of intraluminal phospholipids was not essential for TICE. However, the addition of phospholipids did have a stimulatory effect on TICE.

POSSIBLE FACTORS INVOLVED IN TRANSINTESTINAL CHOLESTEROL EFFLUX

To gain better insight into this RCT pathway and identify key players in the intestine, the ability to stimulate the rate of TICE may be a helpful tool (Figure 2). Unfortunately, expression analysis of cholesterol-related genes in the small intestine from mice fed with a high-fat diet or treated with PPARδ agonist did not reveal many usual suspects[21,22]. The gene expression of the key factor in cholesterol synthesis, i.e. 3-hydroxy-3-methyl-glutaryl-CoA reductase (Hmgcr), was unaffected and the expression of most genes encoding cholesterol transporters was either unchanged or even decreased. One transporter that was upregulated in the small intestine of mice that received a high-fat diet was the scavenger receptor class B type I (SR-BI). Although it can mediate uptake from both LDL and HDL, SR-BI is mostly involved in the cellular cholesterol uptake from circulating HDL[26,27]. Considering their well accepted role in RCT *via* the hepatobiliary route, a comparable role for both SR-BI and HDL in TICE is plausible. However, a direct involvement of SR-BI has become debatable since TICE was also increased in mice deficient for SR-BI[21]. Also, *Sr-b1-/-* mice are characterized by elevated HDL-levels[28], but a possible relationship

between the physiologic concentration of HDL and the rate of TICE is questionable. In *Abca1-/-* mice, which are characterized by low HDL levels and unaltered biliary cholesterol secretion, no decrease in fecal neutral sterol excretion has been observed[29]. In addition, Briand *et al*[30] demonstrated in wild type mice that uptake of HDL by the intestine is significantly lower than the hepatic uptake. Taken together, these data dispute the direct involvement of the major players in the hepatobiliary route. How, and by which cholesterol donor, the cholesterol is transported to the intestine for TICE remains to be elucidated. However, the link that has been established between TICE and deficiency in hepatic cholesterol esterification due to liver-specific depletion of ACAT2 might help to shed some light on this part of the non-hepatobiliary cholesterol secretion route[10].

One important cholesterol transporter that was affected in both high-fat diet-fed and PPARδ agonist-treated mice, was the Niemann-Pick C1-like 1 protein (NPC1L1)[21,23]. Earlier reports could only partially explain the increased fecal neutral sterol output by the reduced cholesterol absorption caused by the attenuated expression of NPC1L1[22,31]. Despite the strong increase in fecal neutral sterol excretion in mice upon treatment with ezetimibe, the inhibitor of NPC1L1, we have shown that NPC1L1 plays no role in TICE[23].

Interestingly, gene expression analysis in the intestine of mice with an increased TICE did reveal two genes encoding proteins that have been associated with the intracellular transport of cholesterol[23]. Rab9 plays a role in cholesterol trafficking from late endosomes to the trans-Golgi network and it has been shown that overexpression of Rab9 can relieve cholesterol build up in Niemann-Pick type C cells, which is a manifestation of a lipid storage disorder[32,33].

The lysosomal integral membrane protein 2 (Limp2) is a lysosomal membrane protein that is known to bind to β-glucocerebrosidase and has specific functions in intracellular vesicular trafficking[34]. However, Limp2 is also the mouse ortholog of the human scavenger receptor class B type 2 (Scarb2) which has been shown to influence cholesterol homeostasis[35]. What the exact relationship between these two factors and TICE might be requires further studies.

Finally, a role for the heterodimer ABCG5/G8 in TICE cannot be ruled out. Despite being a likely candidate for mediating cholesterol secretion into the intestinal lumen in TICE, initial studies in mice lacking functional ABCG5/G8, as mentioned above, only helped in pointing out the existence of TICE. Since TICE was not reduced in *Abcg8-/-* mice, a possible function for ABCG5/G8 in TICE was questionable[9]. However, a more recent study from van der Veen *et al*[19] demonstrated that increase in TICE upon LXR agonist treatment was ABCG5/G8-dependent.

TRANSINTESTINAL CHOLESTEROL EFFLUX: A NEW THERAPEUTIC TARGET

Many cholesterol lowering therapies under development are aimed at increasing cholesterol excretion by stimulation of RCT. Recent studies in mice have now confirmed the presence of direct cholesterol secretion *via* the intestine. The contribution of TICE in mice to cholesterol excretion is even more prominent than that of the biliary cholesterol secretion. Of course, these observations do not constitute the existence and relevance of TICE in humans. However, by the late sixties Simmonds *et al*[36] had already observed cholesterol secretion in the small intestine after performing intestine perfusions in humans. Our group recently estimated that in humans TICE might amount to around one-third of biliary cholesterol secretion, based on average dietary cholesterol intake, biliary cholesterol secretion, and cholesterol excretion in humans[9]. Nonetheless, the exact contribution of TICE in humans still needs to be quantified. Interestingly, it has been documented that the identified and aforementioned stimulators of TICE in mice also have a positive effect on cholesterol excretion in humans. In mice, the secretion of cholesterol by the intestine could be induced by high fat diets and regulated by the phospholipid content in the intestinal lumen[21]. Several studies in men have shown that diets enriched with polyunsaturated fatty acids increase fecal sterol excretion[37-39]. Furthermore, the supplementation of phospholipids strongly stimulated the excretion of sterols in the feces in humans[40].

We are still challenged by a better understanding of the mechanistic details of this novel RCT pathway, but it is clear that TICE presents us with a therapeutic potential in the treatment of atherosclerosis. The direct role of the intestine in the reverse transport of cholesterol from "blood to gut" makes it a suitable and approachable target for cholesterol removal from the body and prevention of cardiovascular diseases.

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TOPIC HIGHLIGHT

Astrid van der Velde, PhD, *Series Editor*

Thyroid hormones and thyroid hormone receptors: Effects of thyromimetics on reverse cholesterol transport

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Abstract

Reverse cholesterol transport (RCT) is a complex process which transfers cholesterol from peripheral cells to the liver for subsequent elimination from the body *via* feces. Thyroid hormones (THs) affect growth, development, and metabolism in almost all tissues. THs exert their actions by binding to thyroid hormone receptors (TRs). There are two major subtypes of TRs, TRα and TRβ, and several isoforms (e.g. TRα1, TRα2, TRβ1, and TRβ2). Activation of TRα1 affects heart rate, whereas activation of TRβ1 has positive effects on lipid and lipoprotein metabolism. Consequently, particular interest has been focused on the development of thyromimetic compounds targeting TRβ1, not only because of their ability to lower plasma cholesterol but also due their ability to stimulate RCT, at least in pre-clinical models. In this review we focus on THs, TRs, and on the effects of TRβ1-modulating thyromimetics on RCT in various animal models and in humans.

Key words: Cardiovascular disease; Cholesterol; Lipoprotein metabolism; Reverse cholesterol transport; Thyroid hormones; Thyroid hormone receptors

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INTRODUCTION

Cardiovascular disease (CVD), resulting from the progression of atherosclerosis, is the leading cause of mortality and is no longer a disease limited to Western countries (for data and statistics visit the World Health Organization homepage at www.who.int). To date, the first treatment choice in the prevention and treatment of CVD are the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, commonly known as statins[1]. These compounds lower hepatic cholesterol levels by activation of sterol regulatory element binding protein 2 (SREBP2). Activation of SREBP2 induces the expression of the low density lipoprotein (LDL) receptor (LDLR), which results in increased uptake of LDL-particles from plasma (see Figure 1 for a schematic representation of lipoprotein metabolism). Newer drugs such as ezetimibe, which acts by blocking intestinal cholesterol uptake, have recently been proposed as complements to statin therapy. Despite these new therapeutic approaches there is still a demand for improved treatment strategies, especially in light of the failure of some clinical trials[2]. A long debated approach is to promote reverse cholesterol transport (RCT)[3]. RCT is a complex process which transfers cholesterol from pe-

ripheral cells to the liver for subsequent elimination in the feces as bile acids and neutral steroids. RCT was originally proposed by Glomset[4] more than 40 years ago. Recently a non-biliary route for cholesterol elimination from the body has been described, named trans intestinal cholesterol excretion (TICE), in which cholesterol can be transported directly from blood across the enterocytes into the intestinal lumen[5].

THYROID HORMONES AND THYROID HORMONE METABOLITES

Thyroid hormones (THs) have prominent effects on growth, development, and metabolism in almost all tissues[6,7]. Thyroxine (T_4) and triiodothyronine (T_3) are synthesized by the thyroid gland and T_4 is the major secreted hormone. Yet, T_3 is classically considered as the active and more potent hormone since it binds to thyroid hormone receptors (TRs) with higher affinity than T_4. Selenoproteins known as deiodinases[8] convert T_4 to T_3 by 5' deiodination of the outer ring of molecules and regulate the local and systemic availability of T_3. Different types of deiodinases exist: type Ⅰ are present in peripheral tissues including the liver; type Ⅱ are mainly present in the pituitary gland, brain, and brown adipose tissue; and type Ⅲ are present in the placenta, brain, and skin. Whereas type Ⅰ deiodinases convert T_4 in the majority of circulating T_3, type Ⅱ deiodinases not only contribute to the circulating levels but also to the intracellular levels of T_3. Thus, type Ⅱ deiodinases confer to the tissues expressing this type of enzyme the ability to respond to circulating T_4 without being obligated to circulating T_3. Type Ⅲ deiodinases, together with type Ⅰ, convert T_4 into reverse T_3 (rT_3). rT_3 was regarded as an inactive metabolite, since no metabolic effects of rT_3 has been reported, however, the discovery of non-genomic actions of rT_3 on actin polymerization and microfilament organization in astrocytes and in the cerebellum[6,9] has shown that this molecule is active. In addition to deiodination, THs are metabolized by sulfation and glucuronidation[10]. These processes primarily occur in the liver, and to a lesser extent in the kidney, and results in relatively inactive metabolites with increased water solubility, which facilitate biliary and urinary secretion. When the activity of type Ⅰ deiodinases is low (e.g. in the fetus), T_3 sulfate may serve as a reservoir of inactive T_3 from which the active hormone can be generated by the action of tissue and intestinal bacterial sulfatases[11]. Similarly, iodothyronine glucuronides once excreted *via* the bile into the intestine can be substrates for the bacterial β-glucuronidases and the unconjugated THs generated can be reabsorbed into the body. Thus, THs undergo enterohepatic recirculation[12].

In the liver, oxidative deamination and decarboxylation of the alanine chain of T_3 and T_4 form triiodothyroacetic acid (Triac) and tetraiodothyroacetic acid (Tetrac), respectively. These so-called acetic acid analogues of THs are metabolically active. Tetrac has been evaluated in patients with myxedema and no major differences in efficacy were reported compared to T_4, except for the need for higher doses of Tetrac[13]. Also for Triac, the therapeutic doses to treat thyroid disorders are higher than those needed for T_4 in order to reach similar thyroid-stimulating hormone suppression[14]. Interestingly, Triac had bigger hepatic metabolic actions without enhanced thyromimetic activity specific to the pituitary gland[14]. The organ-selective effects of Triac are possibly explained by the higher affinity of this acetic acid analogue to TRβ (3.5-fold) and to TRα (1.5-fold) than T_3[15].

THYROID HORMONE RECEPTORS

TRs are members of the large superfamily of nuclear receptors (NRs) and can bind DNA as monomers, homodimers, or heterodimers mainly with the retinoic-X receptor α[16-18]. TRs are ligand-activated transcription factors and bind both THs and TH-response elements (TREs) classically located in the promoter regions of their target genes. TRs have the typical NR structure with a central DNA-binding domain containing two "zinc fingers" motifs which interact with the nucleotide of the TRE-sequences. The ligand-binding domain (LBD) is composed of twelve amphipathic helices, some of which specifically interact with co-activators and co-repressors[19-21]. Upon ligand-activation, TRs modify the conformation of their LBD region; a process that mainly involves helix 12 and results in release of co-repressors (e.g. NCoR and SMRT) and recruitment of co-activators (the steroid receptor co-activator complex[22] and the vitamin D receptor-interacting protein/TR associated protein complex[23]). Due to the interaction with co-repressors, TRs can decrease the transcriptional activity of the target genes, when not ligand-activated by THs. The interpretation of data generated in animal models in which TRs have been genetically depleted require caution when compared to conditions with low levels of circulating THs (e.g. after thyroidectomy, hypophysectomy, or in hypothyroidism). Under these conditions TRs are not ligand-activated and, being still present, may repress transcription. Apart from the genomic effect, which classically are mediated by activation of TRs bound to the promoter region of the target genes, THs may also regulate cells by non-transcriptional mechanisms[7].

The human TRs are encoded by the THRA and THRB genes, located on chromosome 17 and 3, respectively; the two TRα isoforms [TRα1, TRα2 (or c-erbAα2)] are generated by alternative splicing of the TRα mRNA whereas the two TRβ isoforms (TRβ1 and TRβ2) are generated by alternative promoter choice[16,24]. Both TRα1 and TRβ1 are expressed in almost all tissues[25], but the latter is the predominant TR isoform in the liver, brain, and kidney, whereas the former is predominantly expressed in muscle and brown adipose fat. TRβ2 is expressed in the hypothalamus, in the anterior pituitary gland, and in the developing brain[25-27].

LESSONS FROM STUDIES IN RODENTS

The generation of TR specific knock-out mice revealed that the T_3-induced cardiovascular liability is mediated by

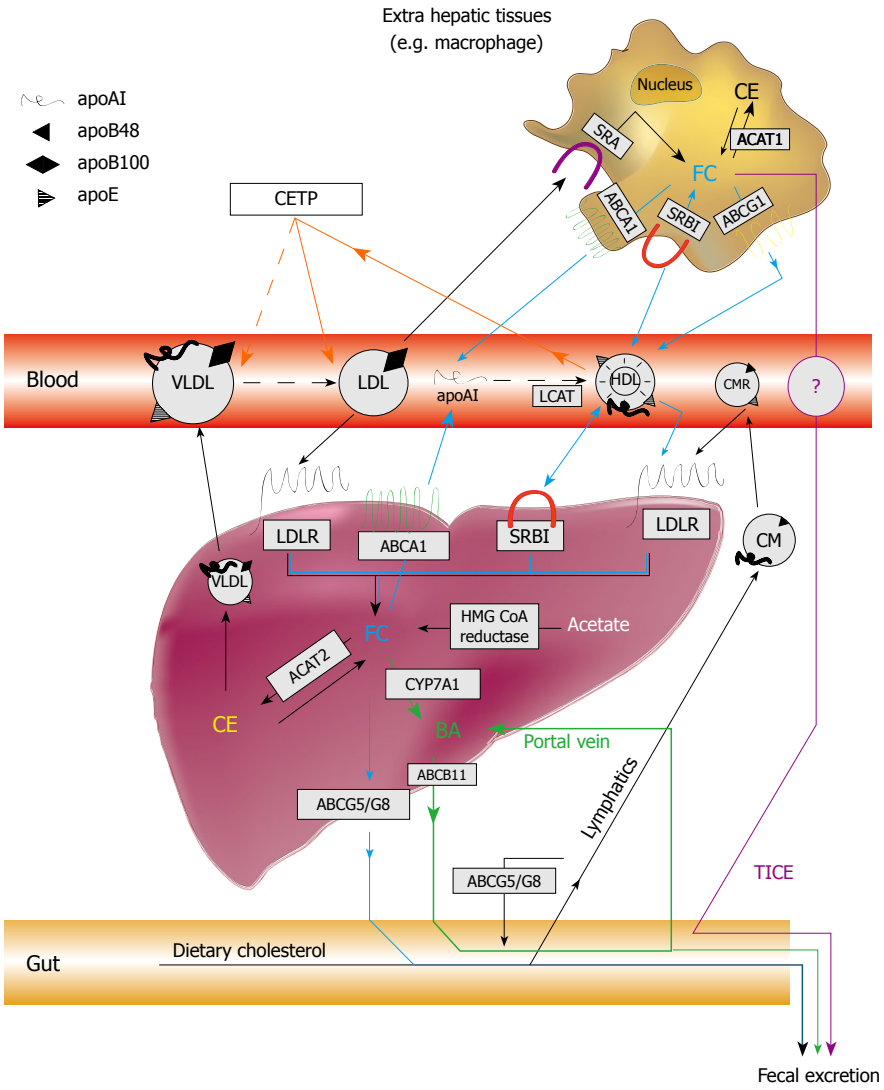

Figure 1 Schematic overview of cholesterol, bile acid, and lipoprotein metabolism. CE: Cholesteryl esters; FC: Free cholesterol; BA: Bile acids; CM: Chylomicrons; CMR: Chylomicron remnants; VLDL: Very low density lipoprotein; LDL: Low density lipoprotein; HDL: High density lipoprotein; apoAI: Apolipoprotein AI; apoB48: Apolipoprotein B48; apoB100: Apolipoprotein B100; apoE: Apolipoprotein E; CETP: Cholesterol ester transfer protein; LCAT: Lecithin cholesterol acyltransferase; LDLR: LDL receptor; ABCA1: ATP-binding cassette transporter A1; ABCG1: ATP-binding cassette transporter G1; ABCG5: ATP-binding cassette transporter G5; ABCG8: ATP-binding cassette transporter G8; ABCB11: ATP-binding cassette transporter B11; SRA: Scavenger receptor type A; SRBI: Scavenger receptor type BI; ACAT1: Acyl-coenzyme A cholesterol acyltransferase 1; ACAT2: Acyl-coenzyme A cholesterol acyltransferase 2; HMGCoA reductase: 3-hydroxy-3-methylglutaryl coenzyme A reductase; CYP7A1: Cholesterol 7α-hydroxylase. Blue lines and arrows represent reverse cholesterol transport. Green lines and arrows represent enterohepatic bile acid circulation. Purple line and arrows represent transintestinal cholesterol excretion (TICE). Red lines and arrows represent the CETP mediated transfer of CE from HDL to LDL and to VLDL.

TRα1[28,29], while the effect of T_3 on plasma cholesterol levels is mediated through TRβ1[30]. These findings raised interest in the development of thyromimetic compounds that specifically modulate TRβ1, either by selective hepatic uptake and/or by higher binding affinity to TRβ1, rather than TRα1. The first thyromimetic compound to be described was SK&F L-94901, which does not preferentially bind to either TRα or TRβ; instead the TRβ1 selective action is achieved by its liver-specific uptake[31]. L-94901 reduced plasma cholesterol levels, mainly in the LDL fraction, in cholesterol-fed hypothyroid and euthyroid rats[32]. Likewise, GC-1 (sobetirome) and KB-141 reduced plasma cholesterol levels in normal and hypothyroid mice and rats[33,34]. T-0681 decreased plasma apoB-containing lipoproteins and reduced atherosclerosis in cholesterol-fed rabbits[35], while MB07811 elicited a similar lipid-lowering effect in rats, as well as in obese mice[36].

The ability of thyromimetic compounds to reduce LDL-cholesterol can partly be explained by increased clearance through increased hepatic LDLR expression. KB-141, MB07811, and T-0681 induced hepatic LDLR expression in several mouse models[35,36], and T-0681 increased hepatic LDLR levels (approximate 2.5-fold) in hypercholesterolemic rabbits[35]. In accordance, LDLR expression was suggested to be crucial for the thyromimetic effect on lipid metabolism, since mice deficient in LDLR do not respond to treatment with either MB07811[36] or T-0681[35]. However, T_3 and sobetirome failed to induce hepatic LDLR mRNA expression and activity, despite reduced circulating levels of LDL-cholesterol in hyper-

cholesterolemic euthyroid mice[34]. Similarly, T-0681 had no effect on the hepatic LDLR protein expression in either C57BL/6 or apoE$^{-/-}$ mice[35]. Thus, the stimulation of LDLR by thyromimetics is not an obligatory finding.

In all animal models, the lipid-lowering effects were achieved at doses that did not affect the heart rate. For sobetirome and KB-141, the concentrations that produced tachycardia were almost 30-fold higher than the therapeutic concentrations in rats and even greater in non-human primates[33].

EFFECTS OF THYROID HORMONES AND THYROMIMETICS ON RCT IN RODENTS

Evidence from animal studies suggested that THs and thyromimetics have the capacity to promote RCT. Despite its complexity, the RCT pathway can be summarized in four major steps: (1) synthesis and lipidation of apolipoprotein AI (apoAI) to generate nascent high density lipoprotein (HDL); (2) efflux of excess cholesterol from peripheral cells (e.g. macrophages) to plasma HDL; (3) hepatic uptake of cholesterol from HDL *via* scavenger receptor class B type I (SRBI) and LDL *via* LDLR - the latter especially in the presence of cholesterol ester transfer protein (CETP); and (4) biliary secretion of cholesterol, as such, or after its conversion to bile acids, for final excretion from the body in feces.

Studies in rodents showed that T_3 and the thyromimetic compound CGS-23425 increased the levels of plasma apoAI[37,38], suggesting that TR stimulation may promote the synthesis of HDL and thus affect the initial step of RCT. Whether thyromimetic compounds stimulate cholesterol efflux to HDL by a direct action on peripheral cells (e.g. macrophages) is still unclear. Studies in rodents show that the ability of THs and thyromimetics to increase RCT is related to their capacity to stimulate the hepatic and final steps of this process by increasing the expression and activity of: (1) SRBI, responsible for the uptake of cholesterol-enriched HDL; (2) cholesterol 7α-hydroxylase (CYP7A1), which converts cholesterol into bile acids in the liver; and (3) ATP-binding cassette transporter G5 (ABCG5) and G8 (ABCG8), which promote biliary cholesterol excretion[30,34-36].

The regulation of bile acid synthesis by TRs and THs has been widely demonstrated in rodents[39-41]. In mice, TRβ has been identified as the primary mediator of the effect of T_3 on the stimulation of CYP7A1 expression and activity[30]. Also thyromimetic compounds such as MB07811, KB-141, T-0681, or sobetirome have been shown to increase the expression of hepatic CYP7A1[34-36]. In addition to the stimulation of bile acid synthesis, we were able to show that sobetirome increases the hepatic SRBI protein expression in normal and hypercholesterolemic euthyroid mice, leading to lower HDL-cholesterol levels and higher fecal bile acid excretion[34]. A limitation of our study was that a direct quantification of the *in vivo* RCT was not performed. RCT can be quantified *in vivo* by assessing the transport of [^{3}H]cholesterol from intraperitoneally injected macrophages to plasma, liver, and feces (called the macrophage-to-feces RCT)[42,43]. Recently, T-0681 was shown to stimulate the *in vivo* RCT in C57BL/6 mice[35] resulting in elevated fecal excretion of radiolabeled cholesterol, both as neutral sterols and as bile acids. This was paralleled by an increase in the hepatic expression of SRBI, CYP7A1, and ABCG5/G8[35].

Mice and rats have no plasma activity of CETP, which transfers cholesteryl esters from HDL to LDL. Thus, the RCT pathway in these rodent models does not properly resemble the human RCT, in which part of the cholesterol originally carried by HDL is delivered to the liver by LDL. Overexpression of human CETP in mice stimulates the *in vivo* RCT and, as expected, a considerable amount of the radiolabeled cholesterol effluxed from the macrophages was transferred from HDL to LDL for subsequent uptake by hepatic LDLR[44]. Surprisingly, T-0681 failed to stimulate *in vivo* RCT in mice overexpressing human CETP[35], despite the stimulation of hepatic SRBI and LDLR. In this mouse model, T-0681 did not affect hepatic ABCG5/G8 and CYP7A1 expression, as observed in wild-type mice[35]. Plasma CETP-mass was reduced and the authors suggested this was a possible cause of disturbed delivery of cholesterol to the liver[35]. Nevertheless, it is evident that it is difficult to draw any definite conclusions relevant to humans by studying mice overexpressing human CETP. In apoE knockout mice, treatment with T-0681 for 8 wk decreased plasma cholesterol levels and reduced the development of atherosclerosis, whereas treatment for 4 wk slightly increased small fatty streak lesions[35]. In line with the above observation, up-regulation of both hepatic ABCG5/G8 and CYP7A1 were only observed after 8 wk of treatment[35]. Recently, we treated (up to 25 wk) apoE-deficient mice with the new thyromimetic compound KB3495 (KaroBio AB). Reduced atherosclerosis and increased fecal excretion of neutral and acidic sterols were observed independently of the circulating levels of cholesterol in apoB-containing lipoproteins. This suggests that stimulation of RCT was *per se* sufficient to achieve the antiatherogenic effects[45]. Furthermore, no major effects on the hepatic expression of ABCG5/G8 mRNA were seen suggesting that TRβ1 modulation may increase RCT possibly by stimulation of TICE[5].

LESSONS FROM STUDIES IN HUMAN AND PRIMATES

It has been known since 1930 that hyperthyroidism is associated with reduced plasma cholesterol levels[46]. Also, studies have shown that hyperthyroid women have lower HDL-cholesterol and apoAI levels compared to healthy controls[47,48]. In addition, treatment with 1-thyroxine in patients with severe primary hypothyroidism significantly increased apoAI but modestly decreased HDL-cholesterol levels[49]. Interestingly, subjects with resistance to thyroid hormone, defined genetically by mutations in TRβ, have lower HDL-cholesterol levels compared to controls[50].

So far, no human or non-human primate studies that specifically aimed to investigate the role of thyromimetics in RCT have been performed. Rodents, unlike humans,

transport plasma cholesterol mainly in HDL-particles, lack CETP activity in plasma, and do not develop atherosclerosis. Also, the feed-forward response on Cyp7A1 activity by dietary cholesterol, which is mediated by activation of liver X receptor α (LXRα) in mice, is absent in humans, because functional LXRα response elements are not present within the human CYP7A1 promoter[51]. Hence, caution is required when extrapolating mechanisms in RCT from rodent studies to humans.

EFFECTS OF THYROMIMETICS ON BILE ACID SYNTHESIS IN HUMANS

Bile acid synthesis serves as the major elimination route of excess cholesterol, participating in maintenance of cholesterol homeostasis and in the hepatic part of RCT. In the liver, cholesterol is converted to 7α-hydroxycholesterol by the microsomal enzyme CYP7A1, the rate-limiting enzyme of the classic pathway, which is then converted to 7α-hydroxy-4 cholesten-3-one (C4). In humans, the classic pathway is responsible for the main part of bile acid synthesis[52]. Thus, it has been shown that plasma levels of C4 reflect bile acid synthesis and that plasma levels of C4 correlate with the enzymatic activity of CYP7A1 assayed in human hepatic microsomes[53-56].

Studies in human hepatoma cells and in primary human hepatocytes suggest that human CYP7A1 expression and promoter activity is actively repressed in response to THs[57,58], suggesting that THs and thyromimetic compounds would decrease bile acid synthesis. Nevertheless, treatment of moderately overweight and hypercholesterolemic subjects with eprotirome (KB2115), administered at 100 and 200 μg orally once daily for 2 wk, increased bile acid synthesis (C4) by approximate 50% and 100%, respectively. Since no effect on cholesterol synthesis in the body (indirectly measured as the ratio of lathosterol to cholesterol in plasma)[59] was observed it seems that eprotirome may induce a net cholesterol efflux from the body[59].

EFFECTS OF THYROMIMETICS ON HDL, apoAI, apoB AND LIPOPROTEIN (a) IN HUMANS AND NON-HUMAN PRIMATES

Measurement of apoAI, the major apolipoprotein in HDL, is as important as the measurement of HDL-cholesterol and the balance between apoB and apoAI (i.e. the apoB/apoAI ratio) indicates cardiovascular risk[60]. In a study by Ladenson *et al*[61], patients with hypercholesterolemia, who were already receiving simvastatin or atorvastatin, were administered 25, 50 or 100 μg eprotirome (KB2115) or placebo daily for 12 wk in addition to continued statin-therapy[61]. Serum total-, LDL-, and HDL-cholesterol, as well as apoB, apoAI, apoB/apoAI ratio, TG, and lipoprotein (a) [Lp(a)] decreased in the eprotirome-treated subjects[61] without adverse effects on heart or bones. In the study by Berkenstam *et al*[59], treatment with eprotirome was found to reduce serum total- and LDL-cholesterol levels as well as the apoB/apoAI ratio without detectable effects on the heart[59]. No significant changes in HDL-cholesterol, TG, Lp(a), or body weight were observed[59]. The discrepancies between these two studies with regard to HDL-cholesterol and apoAI, and whether the combination-therapy with statins and eprotirome[61] affects this, needs to be further investigated by studying CETP and lecithin cholesterol:acyltransferase (LCAT) activities, C4, hepatic gene expression (e.g. SRBI, CYP7A1, ABCG5/G8, ABCA1), and by studies on sterol fecal excretion.

Intestinal and hepatic ABCA1 regulates HDL levels[62,63]. Co-transfection experiments, performed in human embryonal kidney cells (HEK293) with the human ABCA1 promoter and an expression vector for TRβ, showed suppression of the ABCA1 promoter activity in the presence of T_3[64]. Whether TRβ1-modulators suppress the hepatic and intestinal ABCA1 transcription and expression *in vivo* in humans remains to be elucidated.

Lp(a) may contribute to the development of atherosclerosis, and extreme levels have been shown to increase the risk for myocardial infarction[65]. Cynomolgus monkeys have a lipoprotein cholesterol profile that resembles the human profile and express Lp(a). Sobetirome and KB141 reduce plasma levels of Lp(a) in this non-human primate model[33]. Eprotirome in combination with statin-treatment reduced the levels of Lp(a)[61] which was not observed in patients treated with eprotirome only[59], suggesting again that a possible synergism between statins and eprotirome may exist.

CONCLUSION

Compounds that specifically target TRβ1 have consistently been shown to stimulate RCT and decrease atherosclerosis in animal models, and may hypothetically be useful as a complement to statin therapy in the prevention of CVD. However, future studies evaluating the effects of these compounds on RCT in humans need to be performed. Clarification of the primary effect of TRβ1 modulation on human RCT is of great scientific value and strategic interest. The attractiveness of drugs able to promote RCT and lower LDL-cholesterol in humans - especially if not only acting *via* stimulation of LDLR - is immense.

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ORIGINAL ARTICLE

Multiplex RT-PCR-based detections of CEA, CK20 and EGFR in colorectal cancer patients

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Abstract

AIM: To develop a multiplex reverse transcription polymerase chain reaction (RT-PCR) method detecting circulating tumor cells in the peripheral blood of colorectal cancer (CRC) patients.

METHODS: Peripheral blood samples were collected from 88 CRC patients and 40 healthy individuals from the blood donors' clinic and subsequently analyzed by multiplex RT-RCR for the expression of carcinoembryonic antigen (CEA), cytokeratin 20 (CK20) and epidermal growth factor receptor (EGFR) mRNA. The analysis involved determining the detection rates of CEA, CK20 and EGFR transcripts *vs* disease stage and overall survival. Median follow-up period was 19 mo (range 8-28 mo).

RESULTS: Rates of CEA, CK20 and EGFR detection in CRC patients were 95.5%, 78.4% and 19.3%, respectively. CEA transcripts were detected in 3 healthy volunteer samples (7.5%), whereas all control samples were tested negative for CK20 and EGFR transcripts. The increasing number of positive detections for CEA, CK20 and EGFR transcripts in each blood sample was positively correlated with Astler-Coller disease stage ($P < 0.001$) and preoperative serum levels of CEA ($P = 0.029$) in CRC patients. Data analysis using Kaplan-Meier estimator documented significant differences in the overall survival of the different CRC patient groups as formed according to the increasing number of positivity for CEA, CK20 and EGFR transcripts.

CONCLUSION: These data suggest that multiplex RT-PCR assay can provide useful information concerning disease stage and overall survival of CRC patients.

Key words: Peripheral blood; Carcinoembryonic antigen; Cytokeratin 20; Epidermal growth factor receptor; Multiplex reverse transcription polymerase chain reaction

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies in the Western world. In Europe, CRC is the second most frequent cause of death from cancer, after lung cancer[1]. Although surgical resection followed by chemotherapy is the leading treatment option, approximately half of the patients eventually die due to distant metastases[2]. Overall survival is associated with the disease stage at the time of diagnosis, suggesting that early detection of disseminated disease may be of great significance[3]. Consequently, the development of new diagnostic methods that allow better definition of disease stage and better monitoring of disease progression is critical.

In the past years, various techniques have been used for the detection of circulating tumor cells (CTCs) derived from solid tumors in different body compartments, with variable results concerning their accuracy and clinical significance. Reverse transcription polymerase chain reaction (RT-PCR)-based methods have allowed the detection of CTCs in peripheral blood samples, which is characterized by great sensitivity compared to protein-based tumor marker estimations in serum[4]. However, controversial data have been reported, mainly associated with the choice of molecular tumor markers, cohort size and patient characteristics, as well as false-positive and false-negative results, caused by different PCR conditions and different sets of primers employed by different laboratories.

Among several molecular markers, cytokeratin 20 (CK20), carcinoembryonic antigen (CEA) and epidermal growth factor receptor (EGFR) are those more frequently used for CTC detection in CRC[5-7]. CK20 is a member of the intermediate filament protein family and a prominent component of the intestinal epithelium. CK20 expression is confined to gastrointestinal epithelium, urothelium, and Merkel cells of the epidermis, as well as malignancies that originate from the aforementioned sites[8-10]. Serum levels of CEA are commonly used in clinical practice for patient monitoring[11-14]. In addition, CEA and CK20 mRNA detection using RT-PCR methods have been widely investigated for the detection of CTCs in CRC patients[15-22]. Furthermore, EGFR has been suggested as a marker that is seldom expressed in hematopoietic cells[23-25], thus detection of EGFR over-expression in CRC patients has been correlated with the advanced clinical stages and disease progression in CRC patients[26-30].

The aim of the present study was to establish a multiplex PCR assay targeting the detection of CEA, CK20 and EGFR mRNAs in a single PCR reaction and to evaluate its possible clinical use in CRC patients.

MATERIALS AND METHODS

Subjects

Eighty eight patients (56 males and 32 females) with biopsy-proven primary CRC were enrolled in the study between June 2007 and June 2009. All patients underwent surgical resection for the primary tumor at the "Saint Savvas" Anticancer Hospital, the "G. Gennimatas" Athens General Hospital and the "Attikon" University Hospital, Athens, Greece. The mean age was 69 years (range 43-88 years). Peripheral blood samples were also collected from 40 healthy volunteers (20 men and 20 women), which were used as negative controls. Informed consent was obtained from all patients and healthy volunteers. The study was approved by the local Ethics Committee and conformed to the ethical standards of the Helsinki Declaration.

Cell lines and tissue samples

HT-29 CRC cells are known to express the 3 molecular markers used in our experiments and have been used in spiking experiments for the definition of the sensitivity of the assay[16,31-37]. HT-29 cells were maintained in Dulbecco's modified Eagle's medium/F-12 (Cambrex, Walkerville, MD, USA), supplemented with 10% heat-inactivated fetal bovine serum (Biochrom, Berlin, Germany) and 100 U/mL penicillin/streptomycin (Cambrex), at 37℃ in a humidified atmosphere of 5% CO_2.

CRC and matched normal adjacent tissue samples were obtained from 3 CRC patients during surgery, with the aim of confirming the expression of CEA, CK20 and EGFR mRNA in CRC specimens. The samples were collected in RNAlater solution (Ambion, Austin, TX) and subsequently homogenized (Ultra-Turrax T25; Thermo Fisher Scientific, Cheshire, UK) in 1 mL Tri Reagent TR-118 (MRC Inc. Cincinnati, OH, USA) at 500 *g*.

Blood sample preparation and RNA extraction

Three milliliters peripheral blood samples were taken preoperatively using a venous catheter into 3 mL EDTA-containing vacutainers (2 samples were obtained from each patient). All blood samples were processed within 4 h of collection. Each blood sample was added to 7.5 mL of Erythrocyte Lysis Buffer (ELB) (containing 155 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$ and 0.1 mmol/L EDTA pH 7.4) and kept on ice for 45 min with occasional mixing by inversion every 5 min, so as to allow erythrocyte lysis. The samples were then centrifuged at 400 *g* for 10 min at 4℃ and the supernatant was discarded. The pellet of nuclear blood cells was resuspended in 5 mL of ELB and kept on ice for an additional 5 min period in order to remove the remaining red blood cells. Next, the pellet was centrifuged at 400 *g* for 10 min at 4℃. The pellet was then homog-

Table 1 Primer sequences used for reverse transcription polymerase chain reaction amplification of target transcripts

Tumor marker	Sense	Antisense	PCR product size (bp)	Ref.
CEA	5'-GGGCCACTGTCGGCATCATGATTG-3'	5'-TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC-3'	131	[38]
CK20	5'-CAGACACACGGTGAACTATGG-3'	5'-GATCAGCTTCCACTGTTAGACG-3'	371	[39]
EGFR	5'-TCTCAGCAACATGTCGATGG-3'	5'-TCGCACTTCTTACACTTGCG-3'	474	[40]

CEA: Carcinoembryonic antigen; CK20: Cytokeratin 20; EGFR: Epidermal growth factor receptor; PCR: Polymerase chain reaction.

enized in 1 mL Tri Reagent TR-118 (MRC Inc. Cincinnati, OH, USA) using a 5 mL syringe. Total cellular RNA was extracted using Tri Reagent TR-118, according to manufacturer instructions. The RNA pellet was diluted in diethylpyrocarbonate treated water; total RNA concentration and purity were determined by UV spectrophotometry (Biospec-nano, Shimadzu Biotech, Kyoto, Japan) and its quality was confirmed by amplification of cDNA for β-actin housekeeping gene.

RT-PCR

cDNA was synthesized using Moloney murine leukemia virus (M-MuLV) reverse transcriptase RNase H$^-$ (Finnzymes, Oy, Finland). Briefly, a mixture containing 1 μg of total RNA, 0.5 μg (25 μg/mL) oligo-dT$_{(18)}$ primer (Fermentas) and nuclease free water in a total volume of 15 μL was heated at 70℃ for 5 min and then chilled in ice for another 5 min. The mixture was supplemented with 0.5 mmol/L deoxynucleotides (HT Biotechnology LTD), reverse transcriptase buffer containing 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, and 10 mmol/L DTT, 40 U Human Placental RNase inhibitor (HT Biotechnology LTD) and finally 200 U M-MuLV reverse transcriptase up to a final volume of 20 μL; it was subsequently incubated at 37℃ for 60 min. Table 1 presents the exact sets of primers used in our study. The sensitivity of the assay was determined in spiking experiments using serial dilutions of HT-29 CRC cells; 10^5, 10^4, 10^3, 10^2, 10, 1 and 0 cancer cells were added to different corresponding tubes, each containing 3 mL of blood taken from the same healthy subject. Spiking experiments were followed by erythrocyte lysis and RNA extraction, as described above.

PCR conditions

Primer pairs were chosen so that the sequences were located at different exons and their specificity was confirmed using NCBI Blast (Table 1). PCR reaction was performed using *Taq* DNA Polymerase (Qiagen, Hilden, Germany) in a final reaction volume of 25 μL containing 1 × CoralLoad PCR Buffer (contains 15 mmol/L $MgCl_2$), 1.67 mmol/L $MgCl_2$ (final concentration), 200 μmol/L each dNTP and 2.5 U *Taq* DNA Polymerase; cDNA volume and primer concentrations varied depending on the marker and the sample examined. For the amplification of tissue sample cDNAs, primer concentrations and cDNA volumes were 0.4 μmol/L and 2 μL, respectively for all markers, while cycling conditions were 94℃ for 3 min; 94℃ for 1 min, 52℃ for 1 min, 72℃ for 1 min (36 cycles); 72℃ for 10 min for CEA and CK20 cDNA amplifications then 94℃ for 3 min; 94℃ for 1 min, 57℃ for 1 min, 72℃ for 1 min (35 cycles); 72℃ for 10 min for the amplification of EGFR cDNA. The concentrations and the cycling conditions concerning PCR reactions in serial dilutions of HT-29 cells for each marker were as follows: for the amplification of CEA cDNA, 0.08 μmol/L of primers and 1 μL of cDNA were used, while the cycling conditions were 94℃ for 3 min, 94℃ for 30 s, 58℃ for 20 s, 72℃ for 30 s (40 cycles); 72℃ for 2 min; similarly, CK20 cDNA was amplified using 0.35 μmol/L of primers and 1 μL of cDNA, and PCR cycling conditions were 94℃ for 3 min; 94℃ for 30 s, 58℃ for 20 s, 72℃ for 30 s (36 cycles); 72℃ for 2 min; finally, the primer concentration used for EGFR assays was 0.4 μmol/L and 2 μL of cDNA added to the PCR mix, while amplification took place at 94℃ for 3 min; 94℃ for 1 min, 50℃ for 1 min, 72℃ for 1 min (39 cycles); 72℃ for 10 min. For the multiplex PCR reactions 2 μL of cDNA were used, adding primer concentrations as follows: CEA 0.06 μmol/L, CK20 0.15 μmol/L and EGFR 0.5 μmol/L. Cycling conditions were 94℃ for 15 min; 94℃ for 30 s, 58℃ for 20 s, 72℃ for 30 s (37 cycles); 72℃ for 10 min. PCR products were analyzed by electrophoresis on a 2% agarose gel followed by ethidium bromide staining and then captured under ultraviolet light in a Kodak EDAS 290 imaging system (Carestream Health, Rochester, NY, USA).

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences Predictive Analytics Software (SPSS PASW Statistics ver. 18.0) (SPSS Inc., Chicago, IL). The potential correlation between the molecular marker expression and the clinical and pathological characteristics of the patients was tested by Spearman's correlation. The overall survival rates were calculated using the Kaplan-Meier estimator and comparisons of survival curves between patient groups were carried out by the log-rank test. Overall survival was defined as the intermediate time interval between sampling and either death or last follow-up. To assess the independent prognostic significance of factors on overall survival, analysis was performed using the Cox proportional hazards regression analysis. A P value < 0.05 was considered to be statistically significant.

RESULTS

After CEA, CK20 and EGFR mRNA expression was

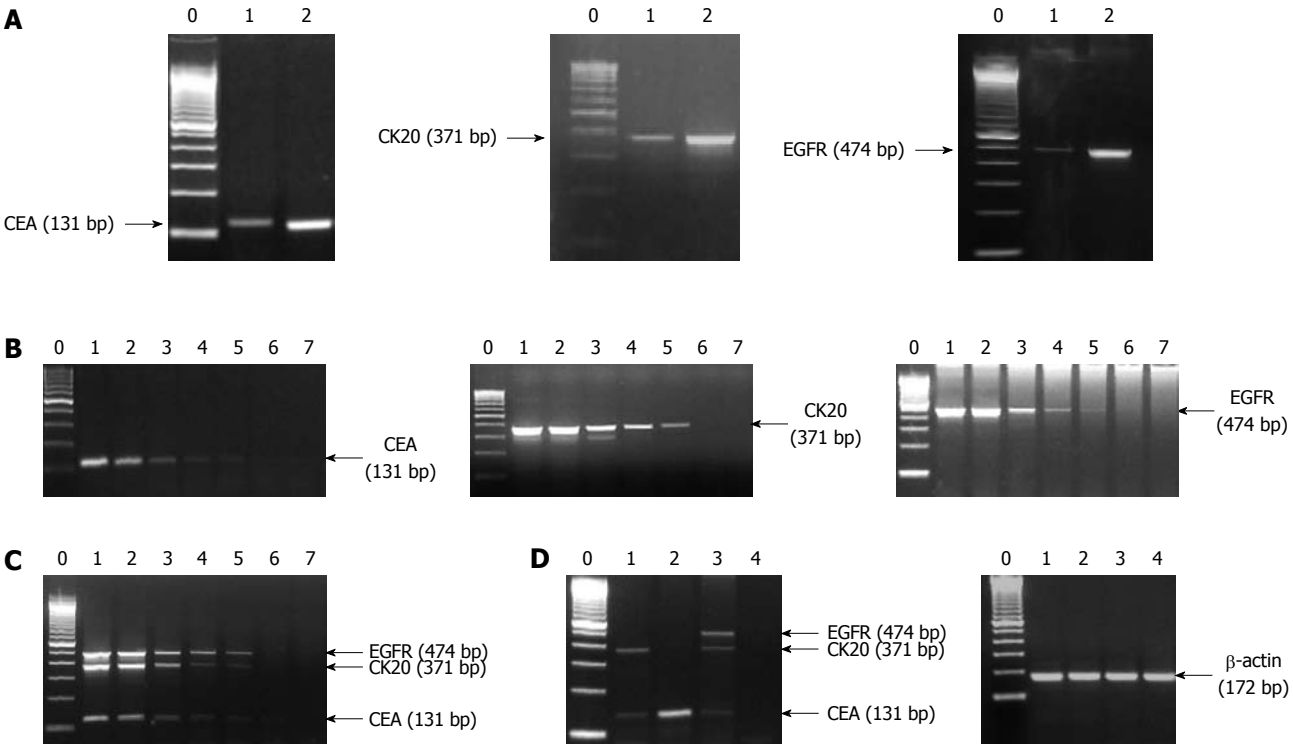

Figure 1 Detection of carcinoembryonic antigen, cytokeratin 20 and epidermal growth factor receptor mRNAs in colorectal cancer tissue samples, HT-29 colorectal cancer cells and colorectal cancer patient peripheral blood samples. A: Detection of carcinoembryonic antigen (CEA), cytokeratin 20 (CK20) and epidermal growth factor receptor (EGFR) mRNA in pairs of colorectal cancer (CRC) and normal adjacent tissue samples. Lane 0 = molecular weight marker (100 bp); lane 1 = normal tissue; lane 2 = CRC tissue; B: Assessment of the sensitivity of the reverse transcription polymerase chain reaction (RT-PCR) detections for CEA, CK20 and EGFR mRNA. Lane 0 = molecular weight marker (100 bp); lane 1 = 10^5 HT-29 cells in 3 mL of normal blood; lane 2 = 10^4 HT-29 cells in 3 mL of normal blood; lane 3 = 10^3 HT-29 cells in 3 mL of normal blood; lane 4 = 10^2 HT-29 cells in 3 mL of normal blood; lane 5 = 10 HT-29 cells in 3 mL of normal blood; lane 6 = 1 HT-29 cell in 3 mL of normal blood; lane 7 = normal blood; C: Assessment of the sensitivity of the multiplex RT-PCR detections for CEA (131 bp), CK20 (371 bp) and EGFR (474 bp) mRNA. Lane 0 = molecular weight marker (100 bp); lane 1 = 10^5 HT-29 cells in 3 mL of normal blood; lane 2 = 10^4 HT-29 cells in 3 mL of normal blood; lane 3 = 10^3 HT-29 cells in 3 mL of normal blood; lane 4 = 10^2 HT-29 cells in 3 mL of normal blood; lane 5 = 10 HT-29 cells in 3 mL of normal blood; lane 6 = 1 HT-29 cell in 3 mL of normal blood; lane 7 = normal blood; D: An example of multiplex RT-PCR-based detection pattern of tumor marker transcripts in the peripheral blood samples of CRC patients. Left: lane 0 = molecular weight marker (100 bp); lane 1 = patient sample positive for CEA (131 bp) and CK20 (371 bp) mRNA; lane 2 = patient sample positive for CEA (131 bp) mRNA; lane 3 = patient sample positive for CEA (131 bp), CK20 (371 bp) and EGFR (474 bp) mRNA; lane 4 = patient sample negative for all markers. Right: lane 0 = molecular weight marker (100 bp); lanes 1-4 = amplification of β-actin (172 bp) cDNA of the patient samples presented in the left side of this panel.

confirmed in pairs of CRC and normal adjacent tissue samples, spiking experiments with HT-29 cells were conducted for establishing the sensitivity of the assay when each marker was run independently (Figure 1). The assay conditions were then accordingly normalized so as to allow the reproducible detection of 10 HT-29 cells in 3 mL of normal blood for all 3 markers examined by multiplex PCR reaction (Figure 1). This cut-off limit has been shown to be of clinical significance in our studies with prostate cancer patients[41-43]. The detection of each marker both in HT-29 cells and in tissue samples (Figure 1, Panel A) was followed by digestion of the PCR product using restriction enzymes (initial development, data not shown). An example of various detection patterns of CEA, CK20 and EGFR transcripts is presented in panel D of Figure 1.

Out of the 40 healthy peripheral blood samples that were examined, 3 were positive for CEA transcripts, resulting in a specificity percentage of 92.5%. We were unable to correlate this finding with any particular clinical information on file of these volunteers. However, both CK20 and EGFR transcripts were absent from the analysis of healthy samples (specificity 100%). CRC patient characteristics are presented in Table 2.

Cox regression analysis was performed by grouping CRC patients according to the number of detected transcripts using our assay, with the aim of comparing the clinical significance of such detection patterns in CRC patients. Six groups of CRC patients were defined: group 0: no marker expression; group 1: positive only for CEA; group 2: positive for CEA and CK20; group 3: positive for all three markers; group 4: patients of groups 1 and 2 and group 5: patients of groups 2 and 3. Disease stage was found to be an independent prognostic factor for survival when comparisons were performed between group 1 and group 2 ($P = 0.005$), between group 2 and group 3 ($P = 0.005$), between group 3 and group 4 ($P = 0.003$) as well as between group 1 and group 5 ($P = 0.002$). When group 1 and group 3 were compared, tumor size was found to correlate significantly with survival ($P = 0.040$); however, the analysis showed a suggestive prognostic significance of other factors, such as lymph node involvement ($P = 0.088$), stage ($P = 0.066$), sex ($P = 0.062$) and the positivity for all transcripts detection ($P = 0.060$). Cox regression

Table 2 Clinical and pathological characteristics of the colorectal cancer patients enrolled in the study

	No. of cases
Total cases	88
Sex	56
Male	32
Female	
Stage (Astler-Coller)	
A	4
B	26
C	27
D	31
Lymph node metastasis	
No	34
Yes	54
Surgical intervention	
Resection	83
No intervention	5
Distant metastasis	
No	57
Yes (liver/lungs/both)	24/2/5
Age (yr), median (min-max)	69 (43-88)
Tumor size (cm), median (min-max)	4.0 (1.0-11.0)

Table 3 Correlation of the number of multiplex reverse transcription polymerase chain reaction positive detections in each blood sample with disease stage in colorectal cancer patients *n* (%)

CRC patients (*n* = 88)	No. of positive marker detections in each blood sample			
	0	1	2	3
Stage A (*n* = 4)	1 (25)	2 (50)	1 (25)	0 (0)
Stage B (*n* = 26)	3 (11.5)	5 (19.2)	16 (61.5)	2 (7.7)
B1 (*n* = 9)	1 (11.1)	1 (11.1)	7 (77.7)	0 (0)
B2 (*n* = 17)	2 (11.8)	4 (23.5)	9 (52.9)	2 (11.8)
Stage C (*n* = 27)	0 (0)	6 (22.2)	17 (63)	4 (14.8)
C1 (*n* = 5)	0 (0)	1 (20)	3 (60)	1 (20)
C2 (*n* = 22)	0 (0)	5 (22.7)	14 (63.6)	3 (13.6)
Stage D (*n* = 31)	0 (0)	2 (6.5)	18 (58.1)	11 (35.5)

Disease staging according to the modified Astler-Coller classification. A positive correlation was revealed using Spearman's test (Spearman's ρ = 0.396, $P < 0.001$). CRC: Colorectal cancer.

analysis could not be performed for the comparison of group 0 with the other groups, since all 4 patients with negative detection for all 3 markers were still alive at the time of analysis.

Our analysis revealed that 84 of 88 CRC patients (95.5%) were positive for CEA transcripts; 69 of 88 (78.4%) were positive for CK20 transcripts and 17 of 88 (19.3%) were positive for EGFR transcripts. In particular, 3 of 4 (75%) stage A; 23 of 26 (88.5%) stage B; 27 of 27 (100%) stage C and 31 of 31 (100%) stage D patients were positive for CEA transcripts. In addition, 1 of 4 (25%) stage A, 18 of 26 (69.2%) stage B, 21 of 27 (77.8%) stage C and 29 of 31 (93.5%) stage D CRC patients tested positive for CK20 transcripts. The EGFR transcripts were detected in 17 of 88 (19.3%) patients: in 2 of 26 (7.7%) stage B; in 4 of 27 (14.8%) stage C and in 11 of 31 (35.5%) stage D. No EGFR transcripts were detected in stage A CRC patients. The correlation of PCR detections to disease stage are presented in Table 3.

We documented that 84 CRC patients (95.5%) were positive by multiplex RT-PCR for at least one transcript (CEA); 69 (78.4%) were positive for at least two transcripts (CEA plus CK20), whereas 17 (19.3%) were positive for all 3 transcripts. Four (4.5%) patients showed no transcript detection. The association between clinical and pathological characteristics of patients with multiplex PCR-based detection of tumor markers was analyzed using Spearman's correlation (Table 4). This analysis revealed that any of the 3 transcript detections was positively correlated with Astler-Coller stage of the disease, when examined singly (CEA: Spearman's $\rho = 0.264$, $P = 0.013$; CK20: Spearman's $\rho = 0.306$, $P = 0.004$; EGFR: Spearman's $\rho = 0.305$, $P = 0.004$). In addition, positive detection of CK20 and CEA transcripts was positively correlated with serum CEA levels (CK20: Spearman's $\rho = 0.285$, $P = 0.014$; CEA: Spearman's $\rho = 0.228$, $P = 0.050$). Furthermore, a positive correlation was evident between positive detection of the three marker transcript panel and Astler-Coller stage (Spearman's $\rho = 0.396$, $P < 0.001$), as well as serum CEA levels (Spearman's $\rho = 0.253$, $P = 0.029$). Moreover, a suggestive (marginal) association was depicted when the positivity for all 3 transcript detections was analyzed *vs* lymph node involvement (Spearman's $\rho = 0.216$, $P = 0.055$) (Table 4).

Interestingly, all CRC patients who tested negative for all 3 tumor transcripts were alive at the time of verification. Of 15 patients who tested positive only for CEA, 14 were alive (93.3%) (follow-up from 9 to 26 mo). Thirty seven of 52 (71.2%) patients who tested positive for both CEA and CK20 were alive while 15 of them (28.8%) had died (follow-up from 1 to 28 mo). However, 10 of 17 patients who tested positive for all 3 transcripts had died (58.8%) and only 7 of them (41.2%) were alive (follow-up from 1 to 23 mo) at the time of analysis.

Kaplan-Meier plots comparing overall survival rates of groups 1, 2, 3, 4 and 5 are shown in Figure 2. Using log-rank test, such analysis revealed statistically significant differences between survival rates of CRC patient groups with different multiplex RT-PCR detection patterns. When patients with positive detection for only one marker (group 1) were compared to those positive for all three markers (group 3), a statistically significant difference in survival rates was observed ($P = 0.002$). Similarly, the comparison between the patient group with positive detections for two markers (group 2) and positive detections for all 3 markers (group 3) revealed significant differences in survival rates ($P = 0.007$). Likewise, significant survival differences were documented when survival of group 4 (groups 1 and 2) was compared to survival of group 3 ($P = 0.001$). In addition, significant differences in the survival of CRC patients were also found when group 1 was compared with group 5 (at least positive for 2 transcripts) ($P = 0.041$). No statistical significance was observed when group 1 was compared to group 2 ($P = 0.116$).

Table 4 Analysis of the clinical and pathological characteristics in relation to the detection pattern of circulating tumor cells using the carcinoembryonic antigen, cytokeratin 20 and epidermal growth factor receptor multiplex assay *n* (%)

Parameter	No. of positive markers in each blood sample				*P*-value
	0	1	2	3	
Age (yr)					
< 69	1 (2.4)	7 (17.1)	26 (63.4)	7 (17.1)	
⩾ 69	3 (6.4)	8 (17)	26 (55.3)	10 (21.3)	0.963
Sex					
Male	3 (5.4)	8 (14.3)	32 (57.1)	13 (23.2)	
Female	1 (3.1)	7 (21.9)	20 (62.5)	4 (12.5)	0.294
Stage (Astler-Coller)					
A + B	4 (13.3)	7 (23.3)	17 (56.7)	2 (6.7)	
C	0 (0)	6 (22.2)	17 (63)	4 (14.8)	
D	0 (0)	2 (6.5)	18 (58.1)	11 (35.5)	< 0.001
Lymph node involvement[1]					
⩽ 3	4 (7.1)	11 (19.6)	35 (62.5)	6 (10.7)	
> 3	0 (0)	4 (16)	14 (56)	7 (28)	0.055
Tumor size (cm)[2]					
< 3	2 (10.5)	2 (10.5)	15 (79)	0 (0)	
3-5	1 (2.5)	8 (20)	21 (52.5)	10 (25)	
> 5	1 (4.8)	5 (23.8)	11 (52.4)	4 (19)	0.774
Differentiation[3]					
Well + moderate	4 (6.8)	10 (17)	36 (61)	9 (15.2)	
Poor + no	0 (0)	4 (22.2)	10 (55.6)	4 (22.2)	0.560
Serum CEA (ng/mL)[4]					
< 5	3 (10)	9 (30)	12 (40)	6 (20)	
5-10	0 (0)	1 (7.1)	11 (78.6)	2 (14.3)	
> 10	0 (0)	4 (13.3)	17 (56.7)	9 (30)	0.029

P-values were calculated by Spearman's test. [1]*P*-value calculation using data from 81 patients (7 missing values); [2]*P*-value calculation using data from 80 patients (8 missing values); [3]*P*-value calculation using data from 77 patients (11 missing values); [4]*P*-value calculation using data from 74 patients (14 missing values). CEA: Carcinoembryonic antigen.

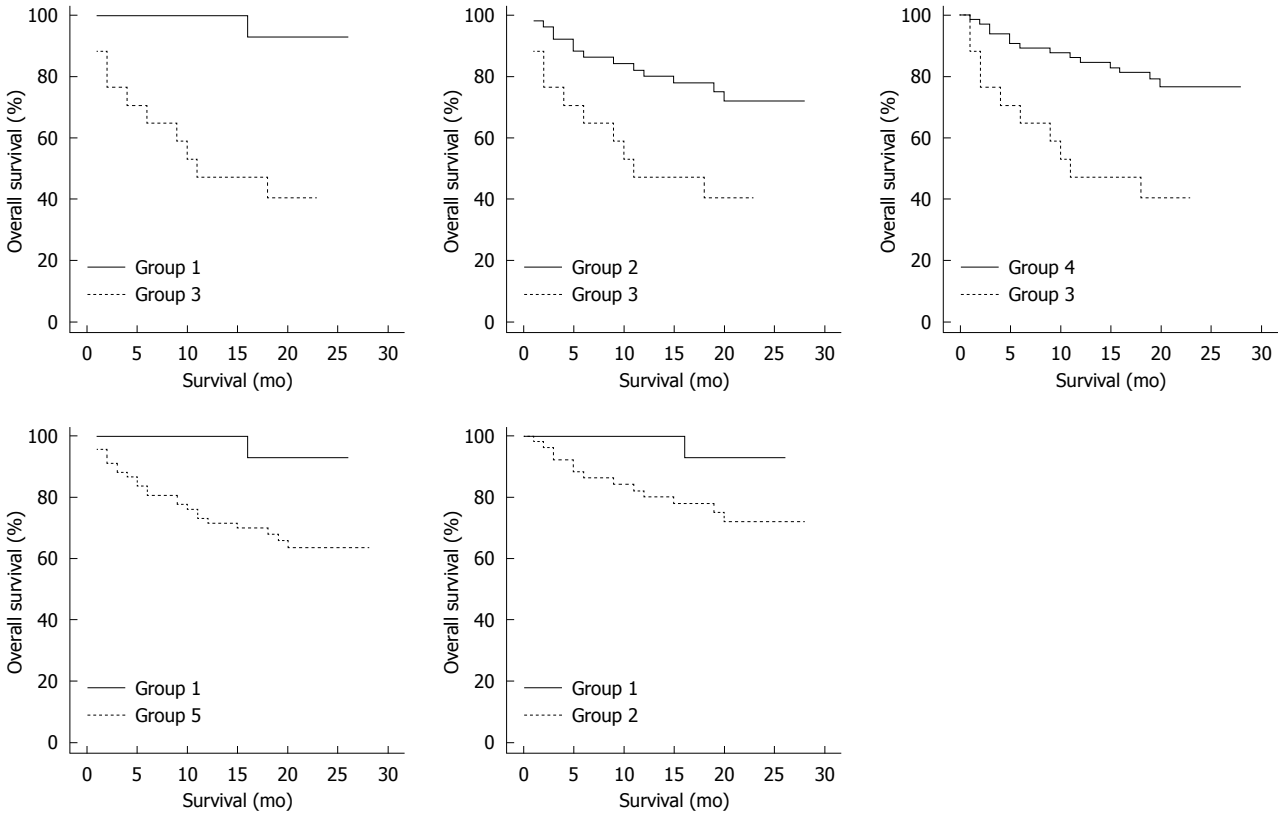

Figure 2 Kaplan-Meier analysis comparing the overall survival of patient groups as developed according to transcript detection patterns for carcinoembryonic antigen, cytokeratin 20 and epidermal growth factor receptor. Group 1: Carcinoembryonic antigen (CEA) positive; Group 2: Positive for 2 markers (CEA and cytokeratin 20); Group 3: Positive for all markers; Group 4: Combination of groups 1 and 2; Group 5: Combination of groups 2 and 3. Log Rank test *P* values were as follows: Groups 1 *vs* 3: *P* = 0.002; Groups 2 *vs* 3: *P* = 0.007; Groups 4 *vs* 3: *P* = 0.001; Groups 1 *vs* 5: *P* = 0.041; Groups 1 *vs* 2: *P* = 0.116.

DISCUSSION

The advantages of multi-marker use for CTC detection in CRC patients have been previously described[5,44-46]. Several studies have shown that the use of more than one marker independently increases the sensitivity and the specificity of CTC detection which correlates with disease stage in CRC patients[47-51]. However, the application of separate PCR reactions for each marker can be impractical, time-consuming and costly. Conversely, the implementation of multiplex RT-PCR which allows the examination of multiple marker expression in a single reaction could be advantageous. The employment of multiplex RT-PCR on peripheral blood samples represents an easily applied, non-invasive technique for the detection of CTCs in cancer patients[52].

The aim of the present study was to develop first and then to test the clinical significance of a multiplex RT-PCR-based detection of three markers (CEA, CK20 and EGFR) in CRC patients. Since we used a short follow-up period, our main analysis was based on overall survival and not on progression-free survival.

Our multiplex PCR-based detections of CEA, CK20 and EGFR transcripts were characterized by very good specificity, since none of the 40 healthy donor samples was found positive for the expression of CK20 or EGFR mRNA and only 3 control samples tested positive for CEA transcripts. This revealed that our assay was of higher specificity than that of previous studies[30,31,53,54]. The observed false positive results in control subjects could be attributed to illegitimate transcription of CEA in mononuclear blood cells. Indeed, it has been reported that CEA mRNA is detected in peripheral blood CD34+ progenitor cells[55]. Based on our data, CEA detection was the most sensitive (expressed in 95.5% of the CRC patients); the positivity rate was higher than that observed by Guadagni *et al*[22] or by Ferroni *et al*[56]. This, however, may be due to clinicopathological characteristics of the enrolled patients in this study and our experimental setting. Moreover, our data suggested that EGFR is possibly a marker for advanced stage in CRC (expressed in 19.3% of the patients and mostly in advanced disease). CK20 detection corresponded to a detection rate of 78.4%, higher than that reported previously by other groups[15,31,57,58]. Again, these detection rate discrepancies among studies using the same mRNA markers can be attributed to patient characteristics, as well as to the particular experimental conditions used by each research group.

Herein, our results revealed that the increasing number of the transcripts detected in this experimental setting could distinguish certain patient groups having significantly different overall survival. However, the detection of 1 (CEA) *vs* the detection of 2 markers (CEA plus CK20) did not show any statistically significant difference in overall survival ($P = 0.116$), which may be attributed to the short follow-up period. Notably, none of the CRC patients who tested negative for all 3 markers died during follow-up. These results are in concert with the results of Wang *et al*[48], who examined a membrane array-based detection of 4 mRNA markers, including CEA and CK20, in 157 CRC patients and found that the positive detection of all 4 markers (human telomerase reverse transcriptase, CK19, CK20, and CEA) was associated with poor overall survival. Similar results were reported by Uen *et al*[47], analyzing the detection of the same transcripts with progression-free survival in CRC patients.

In addition, Spearman's test revealed a positive correlation of positive detection for all 3 transcripts in our assay with higher disease stage and preoperative serum CEA levels. Furthermore, a suggestive correlation was also evident between positive detection of transcripts and higher level of lymph node involvement, suggesting that the higher number of tumor markers detected as positive by multiplex PCR assay is associated with higher levels of serum CEA and a larger amount of lymph node involvement, which are considered as indicators of advanced disease. This is in concert with the findings of Yeh *et al*[49]. Other studies have also examined the detection of CEA, CK20 or EGFR transcripts, however, using single PCR-based assays and reported that the higher number of positive detections correlated with disease stage[18,22,27,59] and serum CEA levels[38,60,61] or the presence of lymph node metastasis[58,62,63].

Herein, the increased number of positive multiplex PCR-based detections in each blood sample correlated with overall survival in CRC patients. Although the detection of the three marker panel was not found to be an independent prognostic factor of overall survival using Cox regression analysis, the log-rank test documented significant differences in overall survival rates among the patient groups formed according to the number of positive detections in each blood sample of CRC patients.

Nevertheless, our data suggested that our multiplex RT-PCR assay can provide useful information concerning CRC stage and overall survival. The combination of the mRNA markers in a single reaction could be of clinical value in the early detection of disseminated disease and monitoring of CRC patients, as it has been documented in other malignancies[5,41-45,64,65]. Future larger-scale studies and longer follow-up surveys would prove the clinical significance and prognostic importance of multiplex PCR-based detection of CTCs using CEA, CK20 and EGFR in CRC patients.

COMMENTS

Background

Colorectal cancer (CRC) is one of the most frequently diagnosed malignancies in both men and women. Overall survival is associated with the disease stage at the time of diagnosis, suggesting that early detection of disseminated disease may be of great significance. Detection of disseminated tumor cells in the peripheral blood of CRC patients could be promising for the early detection of disseminated disease and monitoring of CRC patients.

Research frontiers

Various techniques have been used for the detection of circulating tumor cells (CTCs) derived by solid tumors; among those, reverse transcription polymerase chain reaction (RT-PCR)-based methods have allowed the sensitive detection of CTCs in peripheral blood samples. Among several molecular markers, cytokeratin 20 (CK20), carcinoembryonic antigen (CEA) and epidermal growth fac-

tor receptor (EGFR) are those more frequently used for CTC detection in CRC using PCR-based methods.

Innovations and breakthroughs

Several studies have shown that the use of more than one marker independently increases the sensitivity and the specificity of the CTC detection in CRC patients. However, the application of separate PCR reactions for each marker can be impractical, while the implementation of multiplex RT-PCR allows the examination of multiple marker expression in a single reaction. The employment of multiplex RT-PCR in peripheral blood samples represents an easily applied, non-invasive technique for the detection of CTCs in cancer patients. The results showed that multiplex RT-PCR assay can provide useful information concerning CRC stage and overall survival.

Applications

The combination of mRNA markers in a single reaction could be of clinical value in the early detection of disseminated disease and monitoring of CRC patients. Future larger-scale studies and longer follow-up surveys would prove the clinical significance and prognostic importance of multiplex PCR-based detection of CTCs using CEA, CK20 and EGFR mRNA in CRC patients.

Terminology

CK20 is a member of the intermediate filament protein family and a prominent component of intestinal epithelium. CEA is a high molecular weight glycoprotein that plays a role in CRC metastasis. EGFR exerts control over normal cell growth and cancer pathogenesis in humans. Their mRNAs can be used as markers for the identification of CTCs in peripheral blood samples of CRC patients.

Peer review

This is a well written and produced paper with an important message. This original paper uses multiplex PCR assay for CK20, CEA and EGFR on blood samples to give information about CRC stage and prognosis. The authors have only studied small numbers of cases but the initial results are very encouraging.

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ORIGINAL ARTICLE

Relationship between COX-2 and cell cycle-regulatory proteins in patients with esophageal squamous cell carcinoma

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Abstract

AIM: To investigate the correlation between cyclooxygenase-2 (COX-2) and cell cycle-regulatory proteins in patients with esophageal squamous cell carcinoma (ESCC).

METHODS: One hundred and two surgically obtained specimens of ESCC were randomly collected. All specimens were obtained from patients who had not received chemo- or radiotherapy prior to surgical resection. Twenty-eight specimens of normal squamous epithelium served as controls. The expression of COX-2, Ki-67, cyclin A and p27 was examined by immunohistochemistry. The Pearson test was used to analyze the relationship between groups.

RESULTS: The protein level of COX-2, Ki-67 and cyclin A was significantly higher in ESCC than in normal squamous epithelium (74.7 ± 61.2 *vs* 30.2 ± 43.4, 64.0 ± 51.6 *vs* 11.6 ± 2.3, 44.2 ± 32.2 *vs* 11.7 ± 5.0, respectively, all $P < 0.01$). In contrast, the protein level of p27 was significantly lower in ESCC than in normal squamous epithelium (182.0 ± 69.0 *vs* 266.4 ± 28.0, $P < 0.01$). In ESCC, COX-2 expression was correlated with T stage, the score of T1-T2 stage was lower than that of T3-T4 stage (55.0 ± 42.3 *vs* 83.0 ± 66.5, $P < 0.05$), and Ki-67, cyclin A and p27 expressions were correlated with the tumor differentiation (43.8 ± 31.7 *vs* 98.4 ± 84.8, 32.0 ± 19.0 *vs* 54.1 ± 53.7, 206.2 ± 61.5 *vs* 123.5 ± 68.3, respectively, all $P < 0.01$). COX-2 expression was positively correlated to Ki-67, cyclin A and negatively correlated to p27 expression in ESCC ($r = 0.270$, 0.233 and -0.311, respectively, all $P < 0.05$).

CONCLUSION: The expression of COX-2 is correlated with tumor cell invasion and is closely related to the cell proliferation in patients with ESCC.

Key words: Esophageal squamous cell carcinoma; Cyclooxygenase-2; Ki-67; Cyclin A; p27; Tumor markers; Immunohistochemistry

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INTRODUCTION

Esophageal cancer is one of the most frequently occurring malignancies and the seventh leading cause of cancer-related deaths in the world[1]. Esophageal squamous cell carcinoma (ESCC) remains the most common tumor histologically worldwide. A high incidence of ESCC has been observed in northern China, whereas adenocarcinoma of the esophagus is increasing in some areas of the United States and other Western countries. The prognosis for patients with locally advanced esophageal cancer treated with the standard approaches of surgery or radiotherapy is poor. Treatment failure is mainly due to both a high incidence of local-regional failure and early systemic dissemination of the disease[2].

Several studies have focused on molecular markers in this cancer to elucidate their relation to clinicopathologic features and prognosis of esophageal carcinoma[3-5]. Cyclooxygenase-2 (COX-2) may be involved in an early stage of squamous cell carcinogenesis of the esophagus and plays a non-redundant role in the regulation of cellular proliferation and tumorigenesis of esophageal epithelial cells[6], meanwhile, a significant correlation of COX-2 inhibitor nonsteroidal anti-inflammatory drugs was found with esophageal cancer[7]. COX-2 expression was also associated with the tumor volume response to radiotherapy in patients with cervical squamous cell carcinoma[8], and the use of COX-2 inhibitors could reduce the risk of bone metastases in stage Ⅱ-Ⅲ breast cancer[9].

Ki-67 is known as an indicator of cell proliferation, which is present in all phases of the cell cycle except in G_0. Moreover, Ki67 expression in localized prostate cancer could often predict postoperative progression[10]. In humans, several types of cyclin, including A-type, B-type, and C-type, have been isolated[11-15]. Cyclin A and B1-2 reach maximum levels in the S-phase and G2. Cyclin D1 over-expression indicated a poor prognosis of ESCC[16]. p27, one of the cell cycle-regulatory proteins, is a negative factor of cell proliferation, which is a cyclin-dependent kinase inhibitor[17,18]. Previously, we found that the p27 expression in the nuclei in well-differentiated ESCC was higher than in the other tumor types[19], and Miyabe *et al*[17] thought that p27 might be the most useful prognostic factor in mucoepidermoid carcinoma. Therefore, a molecular research into cancers has resulted in a better understanding of tumor biologic behavior, which has helped improve the survival of cancer patients, on the other hand, clinical application of immunohistochemical methodology may be cost-effective[20]. To our knowledge, the relationship between COX-2 expression and cell cycle-regulatory protein in ESCC has not been reported to date.

The aim of the present study was to investigate the correlation between COX-2 and cell cycle-regulatory proteins and explore the relationship between proliferative activity of cancer cells and COX-2 in patients with ESCC.

MATERIALS AND METHODS

Patients and specimens

We determined the expression of COX-2, Ki-67, cyclin A and p27 in 102 surgically resected ESCC specimens were formalin fixed and paraffin embedded and in 28 normal esophageal mucosa by immunohistochemical method. One hundred and two patients (86 males and 16 females) with ESCC were treated surgically at the Department of Thoracic Surgery, People's Hospital of Taizhou (Taizhou Medical School, Yangzhou and Nantong University) between August 2005 and September 2007. All the patients underwent subtotal or total esophagectomy and radical lymph node dissection.

Histopathological specimens were fixed in 10% buffered formalin, processed routinely, and embedded in paraffin. All specimens were obtained from patients who had not received chemo- or radiotherapy prior to surgery. All hematoxylin and eosin stained sections were reviewed and reexamined by pathologists. The grade of tumor differentiation was determined according to the classification of the World Health Organization[21], and staging according to the TNM classification of American Joint Committee on Cancer[22].

The patients were 35-76 years of age with a median age of 58.0 years. The location of the tumor was as follows: upper intra-thoracic esophagus in 11 cases (10.8%), middle intra-thoracic esophagus in 55 cases (53.9%), and lower intra-thoracic esophagus in 36 cases (35.3%). There were 32 (31.4%) well-differentiated cases, 50 (49%) moderately-differentiated cases (49%), and 20 (19.6%) poorly-differentiated cases. There were 5 stage Ⅰ cases, 47 stage Ⅱ, 33 stage Ⅲ and 17 stage Ⅳ cases. Normal esophageal mucosal samples were taken from the areas > 5 cm from the cancer, as non-tumor control samples.

Antibodies

The following antibodies were used in this study: rabbit monoclonal antibody anti-human COX-2 (Maixin Biotechnology Co., Ltd., Fuzhou, China); mouse monoclonal antibody anti-human Ki-67, p27 and PV-9000 test kit (Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China); mouse monoclonal antibody anti-human cyclin A (Thermo Fisher Scientific, CA). The final diluted concentration was 1:75.

Immunohistochemical staining

The specimens of adjacent non-cancerous esophageal mucosa were cut into 4-5-μm thick sections and mounted onto slides, deparaffinized with xylene, and re-hydrated with graded concentrations of ethanol. Endogenous peroxidase activity was blocked with hydrogen peroxide (H_2O_2) in deionized water for 10 min. The slides were

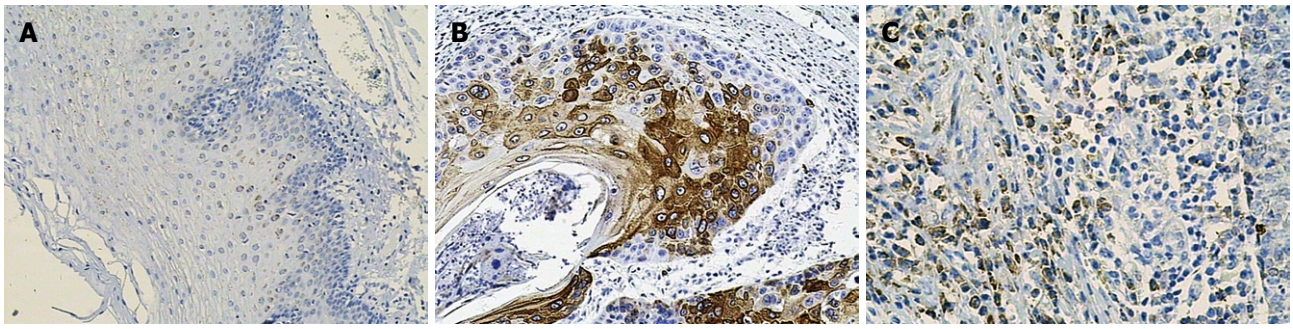

Figure 1 Expression of cyclooxygenase-2 in normal esophageal mucosa and esophageal squamous cell carcinoma. Positive staining in cytoplasm in normal mucosa (A), well-differentiated squamous cell carcinoma (SCC) (B) and poorly-differentiated SCC (C). Counterstaining with hematoxylin, × 200.

washed three times with Tris buffered saline (TBS) buffer (10 mmol/L Tris-HCl, 100 mmol/L NaCl, pH 7.5) for 2 min. An antigen retrieval technique was used before application of the primary antibody (10 mmol/L sodium citrate solution, pH 6.0 in a rice cooker, at 640 W for 30 min). After three washes with TBS, an aliquot of 100 μL of primary antibody was applied to each section and incubated at 4℃ overnight. After washing 3 times with TBS, agent one and then agent two (including the kit) were applied for 20 min at room temperature. Finally, the sections were washed 3 times with TBS, and the immunoreactions were visualized with 0.0067% diaminobenzidine as the substrate with 0.03% H_2O_2 in 100 mmol/L Tris-HCl buffer for 3 min. The sections were lightly counterstained in Haris hematoxylin solution for microscopic examination. Simultaneously, each section was incubated with TBS instead of the primary antibody as an internal negative control. The positive control included: gastric cancer tissue for COX-2, breast cancer tissue for Ki-67 and p27, carcinoma of tonsil for cyclin A.

The immunostained specimens were analysed by two independent pathologists. Cytoplasm and/or nuclear staining (brown reaction product) was regarded as a positive result for COX-2. The staining of Ki-67, p27, cyclin A (brown reaction product) was confined to the nuclei of the cells. Five fields in each tumor and non-tumor section were evaluated at high power (× 400) to determine the proportion of tumor cells and the staining intensity of the cytoplasm and/or nuclei in the entire sections. A score (0-300) was calculated for each marker by multiplying the intensity (none 0, weak 1, moderate 2, strong 3) by the percentage of expression (range 0-100), as reported previously[23]. At least five fields were observed, the average score in each tumor and non-tumor sections served as the result. To confirm the reproducibility of the results, all sections were scored twice, and the highest score from the two observers were thus reported.

Statistical analysis

The frequency of COX-2, Ki-67, cyclin A and p27 staining was expressed as mean ± SE. The correlations between the expression of these biologic molecular and clinicopathological factors were determined using *t* test (two groups had a same range of variance) and analysis of variance. The Pearson test was used to analyze the relationship between groups. SPSS 16.0 (SPSS Inc., USA) software package was used for statistical analysis, and *P* values less than 0.05 were considered statistically significant.

RESULTS

Expression pattern of COX-2 in normal human esophageal mucosa and ESCC

The mean of COX-2 staining in normal esophageal mucosa and ESCC was 30.2 and 74.7, respectively. This result suggested that the expression level of COX-2 in ESCC was higher than in normal human esophageal mucosa, with a significant difference ($t = 5.042$, $P < 0.01$, Figure 1).

Expression pattern of Ki-67, cyclin A and p27 in normal human esophageal mucosa and ESCC

Ki-67 and cyclin A were only expressed in base cells of normal esophageal mucosa. The mean staining rate of the two markers was significantly higher in ESCC (64.0 and 44.2) than in normal esophageal mucosa (11.6 and 11.7, $t = 5.272$ and $t = 5.021$, respectively, $P < 0.01$). The staining of Ki-67 was confined to the nuclei of the cells, while the staining of cyclin A was concentrated mainly in the nuclei of cells, and occasionally in the cytoplasm (Figures 2 and 3). In contrast, p27 staining was observed in all the cells except the base cells of normal esophageal mucosa, the mean staining rates in normal esophageal mucosa and in ESCC were 266.4 and 182, respectively (Figure 4). There was a significantly decreased p27 expression in ESCC ($t = 4.247$, $P < 0.01$).

COX-2, Ki-67, p27 and cyclin A staining and clinicopathological factors

The correlations between the expression of COX-2, Ki-67, p27 and cyclin A and the clinicopathologic features of ESCC are summarized in Table 1. Statistically, the expression of COX-2 was not associated significantly with the age of the patients, tumor size, lymph node metastasis and the TNM stage, but its expression level was significantly higher in T3-T4 than in T1-T2 ($t = 2.62$, $P < 0.05$). For the clinicopathologic features of Ki-67, p27 and cy-

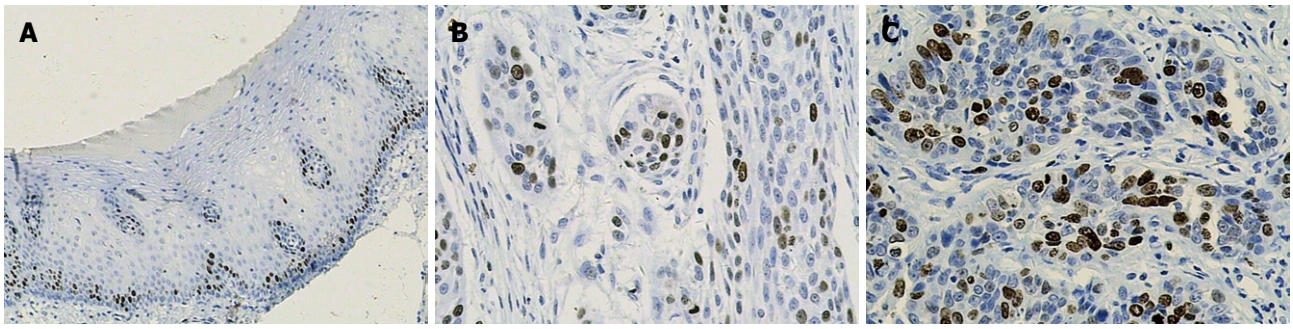

Figure 2 Expression of Ki-67 in normal esophageal mucosa and esophageal squamous cell carcinoma. Positive nuclei staining in normal esophageal mucosa (A, × 100), well-differentiated (B, × 200) and poorly-differentiated squamous cell carcinoma (C, × 200). Counterstaining with hematoxylin.

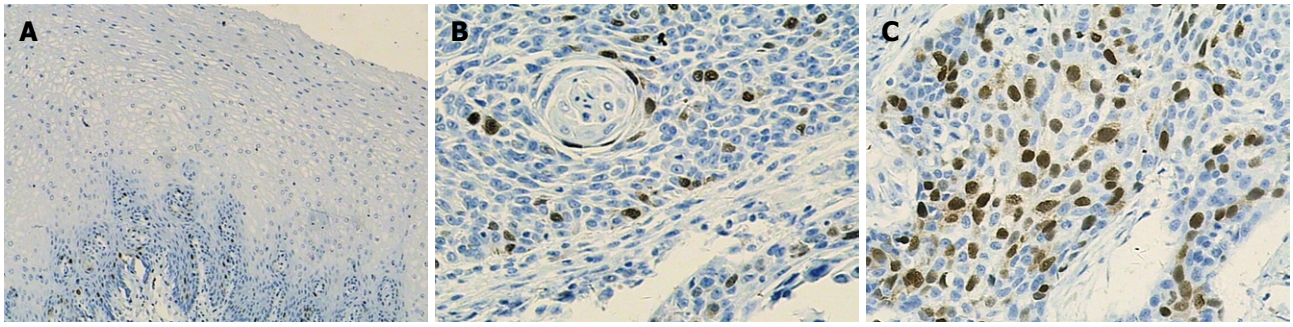

Figure 3 Expression of cyclin A in normal esophageal mucosa and esophageal squamous cell carcinoma. Positive nuclei staining in normal esophageal mucosa (A, × 100), well-differentiated (B, × 200) and poorly-differentiated squamous cell carcinoma (C, × 200). Counterstaining with hematoxylin.

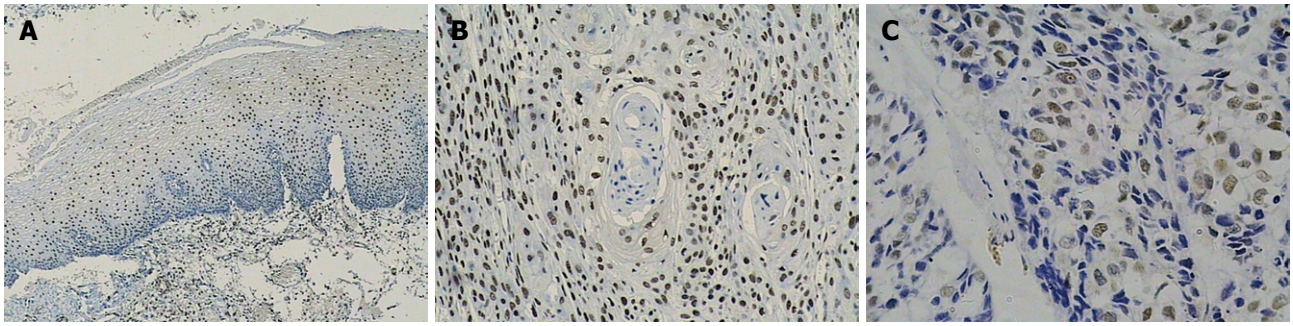

Figure 4 Expression of p27 in normal esophageal mucosa and esophageal squamous cell carcinoma. Sections stained with anti-p27 antibody showing nuclear staining (brown) in normal esophageal mucosa (A, × 100), well-differentiated (B, × 200) and poorly-differentiated squamous cell carcinoma (C, × 200). Counterstaining with hematoxylin.

clin A in ESCC, the expression levels of KI-67 and cyclin A were significantly higher in poorly-differentiated squamous cell carcinoma than in well-differentiated ESCC ($F = 7.839$, $P < 0.01$; $F = 3.519$, $P < 0.05$). The p27 positive immunostaining rate in the nuclei in well-differentiated ESCC was higher than in the other tumor types ($F = 5.49$, $P < 0.01$).

Correlation between COX-2 and cell cycle-regulatory proteins in ESCC

The correlations between the frequency of COX-2 and Ki-67, cyclin A and p27 in ESCC are summarized in Table 2. Positive correlations were observed between the staining frequency of COX-2 and Ki-67 and cyclin A ($r = 0.270$ and 0.233, $P < 0.01$), but negative correlations between the staining frequency of COX-2 and p27 in ESCC ($r = -0.311$, $P < 0.01$).

DISCUSSION

COX-2 expression gradually increased from normal human esophageal mucosa to ESCC. One of the molecular alterations in esophageal carcinomatous change is high COX-2 expression. The staining frequency of COX-2 increased with the tumorigenesis, and its upregulation in ESCC was significantly associated with tumor progression. These results gave additional information about the possibility that COX-2 may be involved in an early stage of squamous cell carcinogenesis of the esophagus[6]. Our study demonstrated that the staining frequency of COX-2

Table 1 Relationship between cyclooxygenase-2, Ki-67, cyclin A, p27 and clinicopathological characteristics of esophageal squamous cell carcinoma

Factors	*n*	COX-2	*P*	Ki-67	*P*	Cyclin A	*P*	p27	*P*
ESCC	102	74.7 ± 61.2	0.00	64.0 ± 51.6	0.00	44.2 ± 32.2	0.00	182.0 ± 69.0	0.00
Normal	28	30.2 ± 43.4		11.6 ± 2.3		11.7 ± 5.0		266.4 ± 28.0	
Age (yr)									
≥ 60	44	77.8 ± 56.4	NS	66.7 ± 48.7	NS	44.6 ± 30.1	NS	185.4 ± 63.3	NS
< 60	58	70.6 ± 67.2		60.5 ± 55.6		43.6 ± 37.2		179 ± 73.3	
Gender									
Male	86	72.8 ± 62.0	NS	64.5 ± 51.3	NS	43.3 ± 32.5	NS	183.6 ± 68.1	NS
Female	16	85.0 ± 57.6		61.5 ± 54.7		48.7 ± 37.3		173.1 ± 67.2	
Location									
Upper	11	59.5 ± 45.9	NS	49.6 ± 27.4	NS	48.3 ± 24.3	NS	205.4 ± 57.8	NS
Middle	55	76.7 ± 62.2		63.3 ± 48.0		45.3 ± 37.0		181.5 ± 67.8	
Lower	36	76.3 ± 64.6		69.6 ± 61.8		41.3 ± 29.7		175.5 ± 74.5	
Differentiation									
Well	32	68.7 ± 48.9	NS	43.8 ± 31.7	0.001	32.0 ± 19.0	0.03	206.2 ± 61.5	0.00
Moderate	50	82.3 ± 63.0		63.2 ± 36.2		47.9 ± 27.8		189.9 ± 61.4	
Poor	20	65.3 ± 74.0		98.4 ± 84.8		54.1 ± 53.7		123.5 ± 68.3	
T stage									
T1-2	32	55.0 ± 42.3	0.028	70.2 ± 46.2	NS	40.6 ± 30.7	NS	169.3 ± 75.1	NS
T3-4	70	83.0 ± 66.5		61.2 ± 54.0		45.8 ± 34.4		187.8 ± 65.8	
N stage									
N0	55	77.9 ± 65.2	NS	63.8 ± 49.8	NS	41.5 ± 28.4	NS	189.8 ± 63.4	NS
N1	47	70.9 ± 56.7		64.3 ± 54.2		47.4 ± 38.1		172.0 ± 74.7	
M stage									
M0	85	74.3 ± 63.2	NS	63.9 ± 52.8	NS	41.8 ± 33.4	NS	181.7 ± 65.1	
M1	17	76.8 ± 52.0		64.7 ± 46.4		54.9 ± 30.4		183.2 ± 75.6	
Overall TNM stage									
Ⅰ	5	62.8 ± 48.3	NS	56.0 ± 34.8	NS	38.4 ± 31.7	NS	201.2 ± 61.5	NS
Ⅱ	48	52.0 ± 40.5		75.7 ± 49.5		41.3 ± 29.6		187.3 ± 66.9	
Ⅲ	32	85.0 ± 66.5		59.3 ± 53.7		43.0 ± 39.4		170.3 ± 71.1	
Ⅳ	17	81.5 ± 67.8		64.5 ± 55.3		55.9 ± 30.4		183.2 ± 75.6	

Upper: Upper intra-thoracic esophagus; Middle: Middle intra-thoracic esophagus; Lower: Lower intra-thoracic esophagus; NS: Not significant; COX-2: Cyclooxygenase-2; ESCC: Esophageal squamous cell carcinoma.

Table 2 Correlation between expression of cyclooxygenase-2 and Ki-67, cyclin A and p27 in esophageal squamous cell carcinoma

Factors	COX-2	Ki-67	Cyclin A	p27
COX-2				
r	1	0.270[b]	0.233[a]	-0.311[b]
P		0.006	0.018	0.001
Ki-67				
r	0.270[b]	1	0.742[b]	-0.292[b]
P	0.006		0.000	0.003
CyclinA				
r	0.233[a]	0.742[b]	1	-0.210[a]
P	0.018	0.000		0.034
p27				
r	-0.311[b]	-0.292[b]	-0.21[a]	1
P	0.001	0.003	0.034	

[a]Correlation is significant (2-tailed, $P = 0.05$); [b]Correlation is significant (2-tailed, $P = 0.01$). COX-2: Cyclooxygenase-2.

was significantly higher in ESCC than in normal esophageal mucosa ($P < 0.01$), suggesting that over-expression of COX-2 was one of the important phenotypes and characteristics in ESCC carcinomatous change.

Apart from the over-expression of COX-2 in patients with ESCC, the present study suggested that tumor with high COX-2 expression was easy to invade. Although there were different conclusions on the relationship between COX-2 and the clinicopathologic features in ESCC, many researchers found that over-expression of COX-2 was associated with infiltrating field of tumor. Nozoe *et al*[24] reported that the prognosis of ESCC patients with a strong COX-2 expression was significantly poorer than that of ESCC patients with a weak COX-2 expression. Based on the data from Liu *et al*[25], the expression of COX-2 was not significantly correlated with gender or age, but increased expression of COX-2 was correlated with invasion and lymph node metastasis statistically. These studies have helped understand the molecular mechanisms of carcinogenesis and progression of ESCC. Yu *et al*[26] suggested that elevated COX-2 expression was not associated with clinicopathological features including age, sex, tumor size, histological grade, lymph node metastasis, and TNM stage. Our study found that expression of COX-2 was associated with T stage (tumor invasion) in ESCC patients, and there was a significantly lower expression in T1-T2 than in T3-T4 ($P < 0.05$)[24,25].

The levels of expression of Ki-67, cyclin A and p27 may suggest the proliferative activity of cancer cells in patients with ESCC. Ki-67, cyclin A over-expression and low p27 expression were associated with poorly-differentiated ESCC, compared with ESCC which had low Ki-67 and cyclin A expression and high p27 expression[3].

Previous studies had revealed the following mechanisms of COX-2 promoting cell proliferation: (1) COX-2 can contribute to inhibition of apoptosis and proliferation, increased angiogenesis, adhesion and invasion and modulation of inflammation; and (2) COX-2 may be involved in an early stage of squamous cell carcinogenesis of the esophagus and plays a non-redundant role in the regulation of cell proliferation and tumorigenesis of esophageal epithelial cells[6,27]. However, these findings are obtained from animal or *in vitro*, and the relationship between COX-2 and tumor cell proliferation in patients with cancer has not been completely elucidated. In this study, the samples were obtained from ESCC patients, and the expression of COX-2 was positively correlated to Ki-67 and cyclin A, which promotes cell proliferation, and was negatively correlated to p27 expression, which is a cell-proliferating suppressor in ESCC. This indicated the higher expression of COX-2 and the stronger tumor cell proliferation in ESCC, and also supported the theory that COX-2 is an important regulatory enzyme in cell proliferation.

In conclusion, our results showed that COX-2 over-expression not only was common in ESCC, but also correlated to tumor cell invasion, and COX-2 had the potential to become a molecular target in the treatment of ESCC. We will investigate the relationship between expression of COX-2 and curative effect of chemotherapy and survival rate of ESCC patients in the future studies. Ki-67 and cyclin A staining was positively correlated while p27 staining was negatively correlated with COX-2 expressions, which suggested that the over-expression of COX-2 was closely related to the cell proliferation in patients with ESCC.

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COMMENTS

Background

Esophageal cancer is one of the most frequently occurring malignancies and the seventh leading cause of cancer-related deaths in the world. Treatment failure results from both a high incidence of local-regional failure and early systemic dissemination of the disease. Cyclooxygenase-2 (COX-2) may be involved in an early stage of squamous cell carcinogenesis of the esophagus and plays a non-redundant role in the regulation of cellular proliferation and tumorigenesis of esophageal epithelial cells. The relationship between COX-2 expression and cell cycle-regulatory protein in esophageal squamous cell carcinoma (ESCC) has not been reported to date.

Research frontiers

COX-2 plays a non-redundant role in the regulation of cellular proliferation and tumorigenesis of esophageal epithelial cells. Ki-67 is known to be an indicator of cell proliferation. Cyclin A reaches maximum levels in the S-phase and G2. p27 is a negative factor of cell proliferation. This study investigated the correlation between COX-2 and cell cycle-regulatory proteins and explore the relationship between the proliferative activity of cancer cells and COX-2 in patients with ESCC.

Innovations and breakthroughs

Many studies have shown recently that not only there was a significant correlation between COX-2 inhibitor nonsteroidal anti-inflammatory drugs (NSAIDs) and esophageal cancer, but also a correlation between the COX-2 expression and the tumor volume response to radiotherapy. Moreover, Ki67 expression, cyclin D1 and p27 might be the most useful prognostic factor in different carcinomas. This study reported that COX-2 expression was positively correlated to Ki-67 and cyclin A and negatively correlated to p27 expression in ESCC. The results may help reveal the molecular feature of ESCC.

Applications

By better understanding tumor biologic behavior, the survival of cancer patients can be improved. COX-2 over-expression not only was common in ESCC, but also correlated to tumor cell invasion, and COX-2 had the potential to become a molecular target in the treatment of ESCC.

Peer review

The authors analyzed the correlation between COX-2 and cell cycle-regulatory proteins in patients with ESCC using immunohistochemistry. They found that COX-2 expression had a significant correlation with T stage and was positively correlated to Ki-67, cyclin A and negatively correlated to p27 expression in ESCC. Considering the samples were from patients, the results are interesting and may help to reveal the molecular feature of ESCC.

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BRIEF ARTICLE

Benefit of combination β-blocker and endoscopic treatment to prevent variceal rebleeding: A meta-analysis

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Abstract

AIM: To determine whether the association of β-blockers with endoscopic treatment is superior to endoscopic treatment alone for the secondary prophylaxis of oesophageal variceal bleeding.

METHODS: Randomised controlled trials comparing sclerotherapy (SCL) with SCL plus β-blockers (BB) or banding ligation (BL) with BL plus BB were identified. Main outcomes were overall and 6, 12 and 24 mo rebleeding rates, as well as overall and 6, 12 and 24 mo mortality. Two statistical methods were used: Yusuf-Peto, and Der Simonian and Laird. Inter-trial heterogeneity was systematically taken into account.

RESULTS: Seventeen randomised controlled trials were included, 14 with SCL and 3 with BL. Combination β-blocker and endoscopic treatment significantly reduced rebleeding rates at 6, 12 and 24 mo and overall [odds ratio (OR): 2.20, 95% confidence interval (CI): 1.69-2.85, $P < 0.0001$] compared to endoscopic treatment alone. Mortality at 24 mo was significantly lower for the combined treatment group (OR: 1.83, 95% CI: 1.16-2.90, $P = 0.009$), as well as overall mortality (OR: 1.43, 95% CI: 1.03-1.98, $P = 0.03$).

CONCLUSION: Combination therapy should thus be recommended as the first line treatment for secondary prophylaxis of oesophageal variceal bleeding.

Key words: Oesophageal varices; Portal hypertension; Cirrhosis; Secondary prevention; β-blockers; Banding ligation

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Funakoshi N, Ségalas-Largey F, Duny Y, Oberti F, Valats JC, Bismuth M, Daurès JP, Blanc P. Benefit of combination β-blocker and endoscopic treatment to prevent variceal rebleeding: A meta-analysis. *World J Gastroenterol* 2010; 16(47): 5982-5992 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i47/5982.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i47.5982

INTRODUCTION

Upper gastrointestinal bleeding from ruptured oesophageal varices is the main complication of portal hypertension and is one of the leading causes of death in patients with cirrhosis. It is estimated that 70% of patients who have survived a first episode of variceal bleeding subsequently rebleed[1]. Secondary prophylaxis of variceal bleeding is thus of utmost importance for these patients.

The use of β-blockers alone has been shown to decrease the risk of recurrent bleeding in cirrhotic patients[2-4]. Endoscopic treatments [sclerotherapy (SCL) and banding ligation (BL)] are also effective in preventing rebleeding in patients with portal hypertension. It has been demonstrated that sclerotherapy decreases the risk of recurrent bleeding and improves survival in cirrhotic patients compared to placebo[5,6]. Banding ligation is currently the endoscopic therapy of choice as it has been shown to be safer and more effective in preventing rebleeding than sclerotherapy[7-10]. However, sclerotherapy continues to be routinely used in a certain number of countries. Combining pharmacological and endoscopic treatment may be more effective than either treatment alone as they act through different mechanisms, thus enhancing each other's therapeutic effect. Many randomised controlled trials have been undertaken to determine whether β-blockers have an additional beneficial role when combined with sclerotherapy or banding ligation to prevent rebleeding. The results of these studies are controversial. Sørensen[11] suggested that β-blockers and sclerotherapy had no additive effect in the prevention of variceal rebleeding, whereas a number of trials have reported favourable results when banding ligation is combined with β-blockers[12,13]. The recommendations of the American College of Gastroenterology[14] state that combined endoscopic and pharmacological therapy is the most effective for secondary prophylaxis, whereas the Baveno IV Consensus conference[15] recommends either β-blockers, banding ligation or both; the authors add that combined therapy is probably the best treatment, but that more trials are needed to prove this. The aim of this meta-analysis is therefore to determine whether the association of β-blockers with endoscopic treatment (sclerotherapy or banding ligation) leads to decreased rebleeding rates and improved survival in patients with portal hypertension compared to endoscopic treatment alone.

MATERIALS AND METHODS

Source of trials

All randomised controlled trials comparing endoscopic treatment alone (sclerotherapy or banding ligation) with a combination of endoscopic treatment associated with β-blockers for the prevention of variceal rebleeding were identified. Trial were retrieved using MEDLINE (1950 to October 2009) and Web of Science (1900 to October 2009), by using the terms "secondary prophylaxis variceal bleeding, endoscopic therapy, β-blockers, sclerotherapy and variceal rebleeding". Conference abstracts from the American Association for the Study of the Liver, European Association for the Study of the Liver, the Digestive Disease Week, the United European Gastroenterology Week and the French National Society of Gastro-enterology between 1980 and 2009 were manually searched. The list of published articles or abstracts was verified and completed through an in depth study of the references quoted in each article. All trials included were peer-reviewed (full articles published in peer reviewed journals or abstracts selected by a peer committee). The final search was performed on April 30, 2010.

Study selection

The trials selected for this meta-analysis met the following criteria: (1) randomised controlled trials published as abstracts, letters to the editor or peer-reviewed articles; (2) patient population: patients presenting portal hypertension (with or without cirrhosis) and oesophageal varices which had previously bled and had not received prior β-blocker or endoscopic treatment; and (3) interventions: treatment with sclerotherapy or banding ligation alone or concomitant treatment with β-blockers (administered from the start of endoscopic treatment) and sclerotherapy or banding ligation; in parallel treatment groups. We chose to exclude studies which associated β-blockers with nitrates or studies in which β-blockers were commenced only after completion of endoscopic therapy. No language restrictions were applied.

Data extraction and quality assessment

Data extraction and quality assessment were performed by three independent reviewers (Ségalas-Largey F, Oberti F and Blanc P). Discrepancies were resolved through discussion before analyses. Trial quality was evaluated using criteria established by Poynard[16] and Nicolucci *et al*[17], simplified and adapted for portal hypertension by Pagliaro *et al*[18]. For each trial, two separate scores were calculated using Poynard's and Pagliaro's criteria; each score was expressed as a percentage of the highest score possible. In addition, we noted for each trial randomisation, investigator blinding, estimation of sample size and intention-to-treat analysis.

Data synthesis and analysis

Primary end-points were all-cause rebleeding rates and mortality at 6, 12 and 24 mo, as well as overall rebleeding and mortality rates as reported at end of follow-up. All-cause rebleeding was defined as upper gastrointestinal bleeding of any source. We chose this as the primary outcome as endoscopic treatment of oesophageal varices may induce bleeding related to oesophageal ulcers. Results were determined at different time points in order to take into account variations in follow-up duration between trials. For each time point, raw data was extracted from studies when available using Kaplan-Meier survival curves.

A meta-analysis including all trials (sclerotherapy and banding ligation) was performed, as well as subgroup analyses of trials using sclerotherapy and trials using banding ligation.

Table 1 Study characteristics

Author	Country	Publication type	*n*	Length of follow-up (mo)
Combination therapy *vs* sclerotherapy				
Westaby *et al*[36]	United Kingdom	Article	53	6
Jensen *et al*[37]	Denmark	Article	31	12
Bertoni *et al*[38]	Italy	Letter	28	2
Gerunda *et al*[39]	Italy	Abstract	60	6
Lundell *et al*[40]	Sweden	Article	41	8
Kanazawa *et al*[32]	Japan	Abstract	43	27
Vinel *et al*[41]	France	Article	74	3
Acharya *et al*[42]	India	Article	114	24
Avgerinos *et al*[43]	Greece	Article	85	23.9
Villanueva *et al*[33]	Spain	Article	40	26
Vickers *et al*[44]	United Kingdom	Article	73	24
Elsayed *et al*[34]	Egypt	Article	178	21
Benedeto-Stojanov *et al*[45]	Yugoslavia	Abstract	65	39
Dowidar *et al*[35]	Egypt	Article	40	16.2
Combination therapy *vs* banding ligation				
Abdel-Rahim *et al*[46]	Egypt	Abstract	50	< 3
Lo *et al*[12]	China	Article	122	12
de la Peña *et al*[13]	Spain	Article	84	16

Statistical methods

All results were expressed as odds ratio (OR), with a 95% confidence interval (CI). Results were first calculated using a fixed-effect model (Collins *et al*[19]). Heterogeneity was calculated using Breslow-Day's test. When significant heterogeneity was found, both the OR and *P*-heterogeneity were subsequently calculated using a random-effect model (DerSimonian *et al*[20]). This model takes into account heterogeneity by providing a more conservative estimate of treatment effect with wider confidence intervals in order to adjust for inter-trial variability. *P*-heterogeneity was initially considered significant if < 0.05; however, when *P*-heterogeneity tended towards significance (i.e. between 0.05 and 0.10), we preferred to take the precaution of calculating results using the random-effect model. The percentage of variability beyond chance was estimated using the I^2 statistic[21]. Publication bias was assessed using the Egger Test[22] and represented graphically using funnel plots[23] plotting the natural log of the OR *vs* its standard error. The Trim and Fill analysis for publication bias was performed using Duval and Tweedie's methods[24]. Additionally, the failsafe number according to Orwin's formula[25] was calculated, which represents the number of non-significant studies which would be necessary to reduce the effect size to a non-significant value. All analyses were performed using Comprehensive Meta-analysis software (version 2.2.048 New Jersey, USA, 2008).

RESULTS

Study identification and selection

Our search identified 229 potentially relevant references. Two hundred and six reports were excluded when it was obvious they did not meet inclusion criteria. A total of 23 reports were considered for detailed analysis. Four studies were excluded as they associated β-blockers with nitrates[26-29]. Two studies were excluded as they started β-blocker therapy after completion of endoscopic therapy[30,31]. Seventeen studies were included in the meta-analysis.

Study characteristics

Study characteristics are reported in Table 1. Fourteen trials[32-45] comparing sclerotherapy with combination sclerotherapy plus β-blockers were included with a total of 925 patients. Among these 925 patients, 887 were analysed: 435 were treated by sclerotherapy alone and 452 by sclerotherapy plus β-blocker. Thirty-eight patients from the trial of Elsayed *et al*[34] were not included as they were excluded from the study after randomisation for various reasons (non compliance, loss to follow up, shunt surgery). Ten studies were published as full articles[33-37,40-44], three in abstract form[32,39,45] and one as a letter to the editor[38]. One study was published in Spanish[33].

Three articles comparing banding ligation with combined banding ligation and β-blocker therapy were included, with a total of 256 patients[12,13,46]. Among these 256 patients, 252 were analysed. Four patients from the trial of de la Peña *et al*[13] were excluded after randomisation due to withdrawal (3 patients) or diagnosis of lymphoma (one patient). One hundred and twenty-five were treated with banding ligation alone and 128 with banding ligation plus β-blockers. Two trials were published as full articles[12,13] and one in abstract form[46].

Patient characteristics are shown in Table 2. Chronic liver disease was the most common cause of portal hypertension in all studies and was mainly due to alcohol abuse in 9 trials[12,33,36-41,44], to viral infection in 3 trials[12,42,43] and to schistosomiasis in 2 trials[34,35]. The aetiology of liver disease was not reported in three studies[32,45,46]. In 8 studies, only patients with cirrhosis were included[32,33,37,39-41,43,45]. Most patients had mild or severe liver disease in 9 studies[12,13,34-38,40,44]. However, in the study by Avgerinos *et al*[43], the majority of patients were Child's class A, whereas in 3 trials Child's class C patients were not included[33,39,42]. Severity of liver disease was either incompletely reported

Table 2 Combination therapy *vs* banding ligation

Author	Mean age (yr)	Men (%)	Non cirrhotic portal hypertension (%)	Alcoholic liver disease (%)	Child class A-B-C (%)	Size of varices small-medium-large (%)
Combination therapy *vs* sclerotherapy						
Westaby *et al*[36]	48.6	62	17	43	16-43-41	0-34-66
Jensen *et al*[37]	46.5	87	0	84	26-45-29	3-32-65
Bertoni *et al*[38]	59.1	64	0	57	32-32-36	7-46.5-46.5
Gerunda *et al*[39]	NA	NA	0	50	NA	NA
Lundell *et al*[40]	56.4	54	0	63	22-27-51	NA
Kanazawa *et al*[32]	NA	NA	0	NA	NA	NA
Vinel *et al*[41]	55.7	78	0	89	NA	0-32-68
Acharya *et al*[42]	34.7	85	11	7	60-40-0	0-0-100
Avgerinos *et al*[43]	58.2	72	0	26	74-19-7	8-45-47
Villanueva *et al*[33]	56.8	57.5	0	50	30-70-0	7.5-62.5-30
Vickers *et al*[44]	55.1	59	15	40	26-51-23	7-26-64
Elsayed *et al*[34]	43.0	84	0	NA	55-29-16	NA
Benedeto-Stojanov *et al*[45]	NA	NA	0	NA	NA	NA
Dowidar *et al*[35]	46.0	90	0	0	45-50-5	20-72.5-7.5
Combination therapy *vs* banding ligation						
Abdel-Rahim *et al*[46]	NA	NA	NA	NA	NA	NA
Lo *et al*[12]	52.0	77	0	30	19-47-34	0-42-58
de la Peña *et al*[13]	60.0	75	0	66	15-56-29	21-54-25

NA: Not available.

Table 3 Combination therapy *vs* sclerotherapy

Study	Random-isation	Investigator blinding	Estimate of sample size	Intention to treat analysis	Poynard's quality score (%)	Pagliaro's quality score (%)
Combination therapy *vs* sclerotherapy						
Westaby *et al*[36]	Yes	No	No	No	31	35
Jensen *et al*[37]	Yes	Double blinded	No	No	65	67
Bertoni *et al*[38]	Yes	Single blinded	No	No	54	44
Gerunda *et al*[39]	Yes	No	No	No	19	12
Lundell *et al*[40]	Yes	Single blinded	No	Yes	50	61
Kanazawa *et al*[32]	Yes	No	No	No	19	18
Vinel *et al*[41]	Yes	No	Yes	Yes	73	43
Acharya *et al*[42]	Yes	Double blinded	Yes	Yes	92	96
Avgerinos *et al*[43]	Yes	Single blinded	Yes	Yes	77	83
Villanueva *et al*[33]	Yes	No	No	No	55	67
Vickers *et al*[44]	Yes	Single blinded	Yes	Yes	83	74
Elsayed *et al*[34]	Yes	No	No	No	50	31
Benedeto-Stojanov *et al*[45]	Yes	NA	NA	NA	29	8
Dowidar *et al*[35]	Yes	Single blinded	No	No	54	50
Combination therapy *vs* banding ligation						
Abdel-Rahim *et al*[46]	Yes	NA	NA	NA	8	17
Lo *et al*[12]	Yes	Single blinded	Yes	Yes	81	85
de la Peña *et al*[13]	Yes	No	Yes	No	69	82

NA: Not available.

or not mentioned at all in 5 studies[32,39,41,45,46]. Variceal size was medium or large for the majority of patients in 11 trials[12,13,33,35-38,41-44] and was not reported in 5 others[32,34,39,40,45]. Sclerotherapy was performed with polidocanol in 5 studies[37,38,40-42], ethanolamine in 6 studies[33-36,43,44] and aetoxisclerol in one study[45]. The sclerosing agent was not reported in 2 trials[32,39]. Banding ligation was performed with multi-band ligators at intervals varying from 10 to 21 d, until complete variceal eradication was achieved. Propranolol was the β-blocker used in 12 trials[32,34-37,40-46] and nadolol in 5 trials[12,13,33,38,39]. The time to eradication of oesophageal varices was reported in 13 trials[12,13,33-38,40-44]. In the sclerotherapy plus β-blocker group the mean time to eradication of varices was slightly shorter (166 d-5.7 sessions) than in the sclerotherapy group (171 d-6 sessions). For patients treated with banding ligation, eradication was achieved in 44 d (3.3 sessions) *vs* 42 d (3.15 sessions) in the banding ligation plus β-blocker group. β-blockers were administered from the start of sclerotherapy until eradication in 7 studies[35-41], whereas in 5 studies[32-34,42,44], β-blockers were given both during and following variceal eradication. Follow-up duration for sclerotherapy studies ranged from 2 to 39 mo (mean 16.6 mo). Follow-up duration for banding ligation studies ranged from < 3 to

Table 4 Meta-analysis comparing endoscopic therapy (sclerotherapy or banding ligation) with combined endoscopic and β-blocker therapy

	SCL/BL, *n*/*N* (%)	SCL/BL + BB, *n*/*N* (%)	No. of trials analysed	I^2	OR (95% CI)	*P* for heterogeneity	*P*-value
All-cause rebleeding							
6 mo	121/410 (29.5)	83/428 (19.4)	11	36	1.70 (1.24-2.34)[1]	0.11	0.01
12 mo	142/376 (37.8)	85/392 (21.7)	10	62.3	2.22 (1.25-3.99)[2]	0.004	0.007
24 mo	106/272 (40.0)	80/285 (28.1)	7	50	1.67 (0.99-2.81)[2]	0.08	0.05
Overall	211/537 (39.3)	132/561 (23.5)	16	12.8	2.20 (1.69-2.85)[1]	0.31	< 0.0001
Mortality							
6 mo	39/309 (12.6)	33/319 (10.3)	9	0	1.23 (0.75-2.04)[1]	0.53	0.41
12 mo	33/246 (13.4)	30/259 (11.6)	7	0	1.18 (0.69-1.99)[1]	0.79	0.55
24 mo	58/230 (25.2)	37/244 (15.2)	6	0	1.83 (1.16-2.90)[1]	0.92	0.009
Overall	98/536 (18.3)	76/560 (13.6)	16	0	1.43 (1.03-1.98)[1]	0.97	0.03

[1]Fixed effect model; [2]Random effect model. SCL: Sclerotherapy; BL: Banding ligation; BB: β-blockers; OR: Odds ratio; CI: Confidence interval.

16 mo (mean 10.3 mo). Overall follow-up duration ranged from 2 to 39 mo (mean 15.4 mo).

Quality assessment

Variables defining study quality are presented in Table 3. All trials were correctly randomised. Three trials were double-blinded[12,37,42] and six trials were single-blinded[13,35,38,40,43,44]. Sample size was calculated in six trials. Six studies[12,40-44] were performed on intention-to-treat basis. The quality scores determined by Poynard's criteria and by Pagliaro's criteria were strongly correlated.

Outcome measures

All-cause rebleeding: The all-cause rebleeding rate was reported in all studies except for the trial of Lundell *et al*[40]. For sclerotherapy trials, 162 patients rebleed in the sclerotherapy group (39.2%) whereas 110 patients (25.4%) rebleed in the sclerotherapy plus β-blocker group. Four sclerotherapy trials[32,34,37,41] found rebleeding rates to be significantly lower in the sclerotherapy plus β-blocker group; the difference was not significant for the remaining 10 studies. For banding ligation studies, 49 patients bled in the banding ligation only group (39.5%), whereas 22 patients bled in the banding ligation plus β-blocker group (17.2%). Two banding ligation studies found rebleeding rates to be significantly lower in the banding ligation plus β-blocker group[12,13]. The third study[46] also found bleeding rates to be lower in this group, without specifying whether the difference was significant or not. When sclerotherapy and banding ligation studies were pooled, a total of 211 patients (39.3%) bled in the endoscopic therapy only group, and 132 (23.5%) bled in the endoscopic therapy plus β-blocker group.

When sclerotherapy and banding ligation trials were pooled for meta-analysis (Table 4), rebleeding rates were significantly lower in the endoscopic therapy plus β-blocker group at 6 and 12 mo, and for overall end-of-follow-up bleeding rates (at 6 mo, OR: 1.70, 95% CI: 1.24-2.34, $P = 0.01$; at 12 mo, OR: 2.22, 95% CI: 1.25-3.99, $P = 0.007$; overall OR: 2.20, 95% CI: 1.69-2.85, $P < 0.0001$). At 24 mo, the decrease in rebleeding in favour of the endoscopic plus β-blocker group was at the limit of significance (OR: 1.67, 95% CI: 0.99-2.81, $P = 0.05$). All funnel plots were symmetrical; the failsafe numbers were 8, 16 and 33, respectively, for bleeding rates at 6 and 12 mo and overall. The Egger tests were negative for publication bias ($P = 0.47$, $P = 0.31$, $P = 0.39$, respectively). Forest plots for 12 mo and overall rebleeding rates are shown in Figure 1A and B.

Mortality: Mortality was reported in all studies except for the trial of Kanazawa *et al*[32]. In sclerotherapy trials, 71 patients died in the sclerotherapy group (17.2%), whereas 59 deaths occurred in the sclerotherapy plus β-blocker group (13.7%). In banding ligation trials, 27 patients died in the banding ligation group (21.8%), whereas 17 patients died in the banding ligation plus β-blocker group (13.3%). None of the sclerotherapy or banding ligation trials reported a significant difference in mortality between the two treatment arms. When sclerotherapy and banding ligation studies were taken together, a total of 98 patients died in the endoscopic therapy group (18.3%) and 76 died in the endoscopic therapy plus β-blocker group (13.6%).

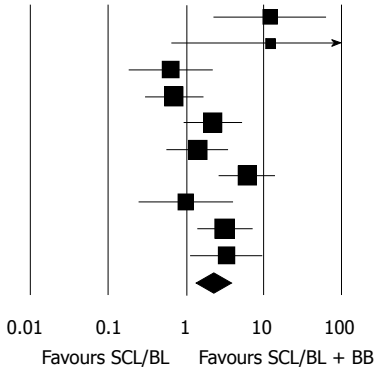
No significant difference in mortality rates was present at 6 and 12 mo. However, at 24 mo a significant difference in mortality in favour of the combination therapy (endoscopy plus β-blocker) group appeared (OR: 1.83, 95% CI: 1.16-2.90, $P = 0.009$). Overall mortality rates were also significantly decreased in the combination therapy group (OR: 1.43, 95% CI: 1.03-1.98, $P = 0.03$), as shown in Table 4. Funnel plots seemed symmetrical, and the failsafe number for both mortality at 24 mo and overall mortality was 2. The Egger test did not find evidence of publication bias ($P = 0.17$ and $P = 0.34$, respectively). Forest plots for overall and 24 mo mortality are shown in Figure 1C and D.

Subgroup analyses

Sclerotherapy trials: A subgroup analysis was performed including sclerotherapy trials only. The rebleeding rates at 6, 12 and 24 mo were lower in the sclerotherapy plus β-blocker group, but the difference was not significant (Table 5). When overall end-of-follow-up rebleeding rates were taken into account, the difference was significantly lower in favour of the sclerotherapy plus β-blocker group (OR: 2.00, 95% CI: 1.49-2.69, $P < 0.0001$). The failsafe number was 54, the funnel plot seemed symmetrical and

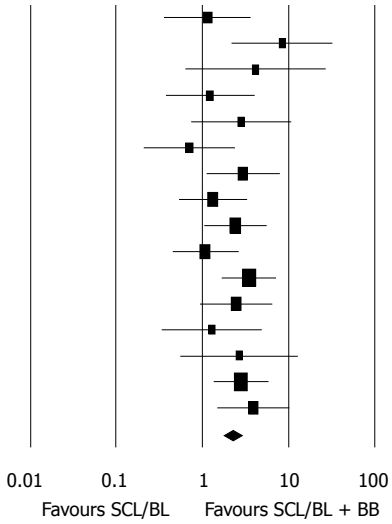
A

Study	MH OR	Lower limit	Upper limit	*Z*-value	*P*-value
Jensen *et al*[37]	12.000	2.198	65.515	2.869	0.004
Kanazawa *et al*[32]	12.189	0.630	235.815	1.654	0.098
Villanueva *et al*[33]	0.636	0.180	2.251	-0.701	0.483
Acharya *et al*[42]	0.701	0.290	1.695	-0.789	0.430
Avgerinos *et al*[43]	2.227	0.910	5.449	1.754	0.079
Vickers *et al*[44]	1.410	0.550	3.614	0.715	0.475
Elsayed *et al*[34]	6.160	2.570	14.769	4.075	0.000
Dowidar *et al*[35]	1.000	0.239	4.184	0.000	1.000
Lo *et al*[12]	3.158	1.350	7.387	2.652	0.008
de la Peña *et al*[13]	3.340	1.117	9.987	2.158	0.031
	2.229	1.249	3.978	2.713	0.007



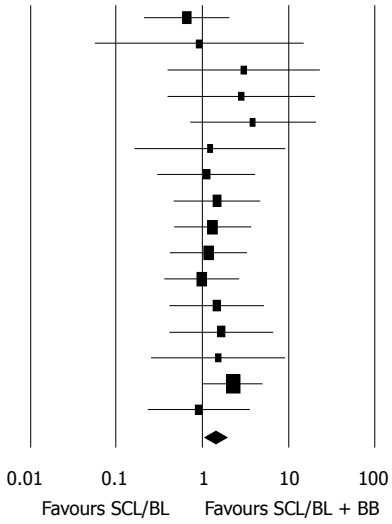
B

Study	SCL/BL	SCL/BL + BB	Peto OR	Lower limit	Upper limit	*Z*-value	*P*-value
Westaby *et al*[36]	8/27	7/26	1.140	0.349	3.725	0.217	0.829
Jensen *et al*[37]	12/16	3/15	8.425	2.106	33.713	3.012	0.003
Bertoni *et al*[38]	4/14	1/14	4.091	0.612	27.332	1.454	0.146
Gerunda *et al*[39]	7/30	6/30	1.213	0.359	4.101	0.311	0.756
Kanazawa *et al*[32]	8/23	3/20	2.759	0.710	10.722	1.466	0.143
Villanueva *et al*[33]	9/18	13/22	0.699	0.203	2.407	-0.568	0.570
Vinel *et al*[41]	14/35	7/39	2.916	1.067	7.970	2.086	0.037
Acharya *et al*[42]	12/56	10/58	1.305	0.517	3.296	0.564	0.573
Avgerinos *et al*[43]	21/40	14/45	2.393	1.012	5.657	1.988	0.047
Vickers *et al*[44]	18/34	20/39	1.068	0.428	2.664	0.141	0.888
Elsayed *et al*[34]	27/70	10/70	3.456	1.635	7.306	3.247	0.001
Benedeto-Stojanov *et al*[45]	16/30	11/35	2.431	0.910	6.489	1.773	0.076
Dowidar *et al*[35]	6/20	5/20	1.277	0.324	5.029	0.350	0.727
Abdel-Rahim *et al*[46]	5/25	2/25	2.656	0.546	12.914	1.210	0.226
Lo *et al*[12]	29/62	14/60	2.769	1.321	5.804	2.698	0.007
de la Peña *et al*[13]	15/37	6/43	3.881	1.438	10.473	2.678	0.007
			2.198	1.693	2.853	5.912	0.000



C

Study	Deaths/total SCL/BL	Deaths/total SCL/BL + BB	Peto OR	Lower limit	Upper limit	*Z*-value	*P*-value
Westaby *et al*[36]	7/27	9/26	0.667	0.209	2.133	-0.682	0.495
Jensen *et al*[37]	1/16	1/15	0.935	0.056	15.703	-0.046	0.963
Bertoni *et al*[38]	3/14	1/14	3.080	0.385	24.627	1.061	0.289
Gerunda *et al*[39]	3/30	1/30	2.868	0.384	21.442	1.026	0.305
Lundell *et al*[40]	5/22	1/19	3.911	0.704	21.742	1.558	0.119
Villanueva *et al*[33]	2/18	2/22	1.245	0.160	9.671	0.209	0.834
Vinel *et al*[41]	5/35	5/39	1.132	0.301	4.261	0.183	0.855
Acharya *et al*[42]	7/56	5/58	1.504	0.457	4.951	0.672	0.502
Avgerinos *et al*[43]	9/40	8/45	1.339	0.464	3.858	0.540	0.589
Vickers *et al*[44]	9/34	9/39	1.197	0.415	3.455	0.333	0.739
Elsayed *et al*[34]	8/70	8/70	1.000	0.354	2.822	0.000	1.000
Benedeto-Stojanov *et al*[45]	6/30	5/35	1.492	0.411	5.423	0.608	0.543
Dowidar *et al*[35]	6/20	4/20	1.682	0.409	6.913	0.721	0.471
Abdel-Rahim *et al*[46]	3/25	2/25	1.546	0.248	9.630	0.467	0.641
Lo *et al*[12]	20/62	10/60	2.302	1.013	5.232	1.991	0.046
de la Peña *et al*[13]	4/37	5/43	0.922	0.232	3.674	-0.115	0.909
			1.430	1.031	1.985	2.140	0.032



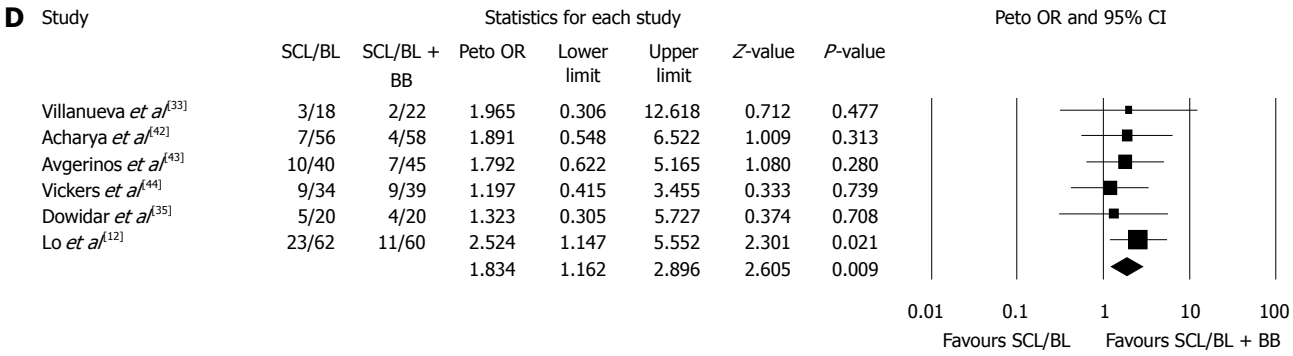


Figure 1 Meta-analysis for rebleeding at 12 mo (A), overall rebleeding (B), overall mortality (C) and mortality at 24 mo (D) for trials comparing combined endoscopic treatment (sclerotherapy or banding ligation) and β-blockers with endoscopic treatment alone. SCL: Sclerotherapy: BL: Banding ligation; BB: β-blockers; OR: Odds ratio; CI: Confidence interval.

Table 5 Subgroup meta-analysis including trials comparing sclerotherapy with sclerotherapy and β-blockers

	SCL, *n/N* (%)	SCL + BB, *n/N* (%)	No. of trials analysed	I^2	OR (95% CI)	*P* for heterogeneity	*P*-value
All-cause rebleeding							
6 mo	91/311 (29.2)	74/325 (22.8)	9	5.8	1.36 (0.95-1.95)[1]	0.39	0.09
12 mo	105/277 (38.0)	69/289 (23.9)	8	69.0	2.03 (0.97-4.25)[2]	0.002	0.06
24 mo	61/173 (35.3)	56/182 (30.8)	5	45.7	1.26 (0.80-2.00)[1]	0.12	0.33
Overall	162/413 (39.2)	110/433 (25.4)	13	20.7	2.00 (1.49-2.69)[1]	0.23	< 0.0001
Mortality							
6 mo	30/247 (12.1)	29/259 (11.2)	8	0	1.07 (0.61-1.87)[1]	0.57	0.82
12 mo	21/184 (11.4)	23/199 (11.6)	6	0	0.98 (0.52-1.85)[1]	0.83	0.96
24 mo	34/168 (20.2)	26/184 (14.1)	5	0	1.56 (0.89-2.73)[1]	0.97	0.12
Overall	71/412 (17.2)	59/432 (13.7)	13	0	1.33 (0.91-1.94)[1]	0.97	0.14

[1]Fixed effect model; [2]Random effect model. SCL: Sclerotherapy; BL: Banding ligation; BB: β-blockers; OR: Odds ratio; CI: Confidence interval.

Table 6 Subgroup meta-analysis including trials comparing banding ligation with banding ligation and β-blockers

	BL, *n/N* (%)	BL + BB, *n/N* (%)	No. of trials analysed	I^2	OR (95% CI)	*P* for heterogeneity	*P*-value
Overall all-cause rebleeding	49/124 (39.5)	22/128 (17.2)	3	0	3.16 (1.76-5.34)[1]	0.85	< 0.0001
Mortality	27/124 (21.8)	17/128 (13.3)	3	0	1.78 (0.92-3.43)[1]	0.53	0.09

[1]Fixed effect model. SCL: Sclerotherapy; BL: Banding ligation; BB: β-blockers; OR: Odds ratio; CI: Confidence interval.

the Egger test did not indicate publication bias ($P = 0.46$). No significant difference in mortality rates was present at 6, 12, 24 mo or with overall end-of-follow-up mortality (Table 5).

Banding ligation trials: A subgroup analysis was performed including the three banding ligation trials (Table 6). Outcomes were significantly in favour of banding ligation plus β-blockers concerning overall bleeding rates (OR: 3.16, 95% CI: 1.76-5.34, $P < 0.0001$). The failsafe number was at 4, and the Egger test did not indicate publication bias ($P = 0.47$). No significant difference was present for mortality. Analyses at different time-points were not performed due to insufficient data.

DISCUSSION

The results of this meta-analysis show that the association of endoscopic treatment with β-blockers is more effective in secondary prophylaxis of oesophageal variceal bleeding than endoscopic treatment alone, with a significant decrease in rebleeding rates overall and at all time points (6, 12 and 24 mo). We have shown for the first time a significant decrease in mortality rates with combined β-blocker and endoscopic treatment over endoscopic treatment alone, with significantly decreased overall and 24 mo mortality rates. Banding ligation is currently the preferred endoscopic treatment for oesophageal varices as it has been shown to be superior to due to less recurrent bleeding, fewer complications, and lower mortality rates[7,9,10,47-49]. However, we chose to include studies using sclerotherapy in our meta-analysis, as it continues to be widely used in the developing world, where schistosomiasis, one of the leading causes of portal hypertension worldwide[50], and viral B cirrhosis are prevalent.

The superiority of combination β-blocker and endoscopic treatment in reducing rebleeding rates may be explained by their synergistic action, with β-blockers reduc-

ing portal hypertension, while sclerotherapy and banding ligation act focally on the oesophagus. Although overall rebleeding rates are significantly in favour of combination therapy for sclerotherapy trials alone, when studies using banding ligation are added to those using sclerotherapy, the decrease in rebleeding rates in the combination therapy group becomes significant at 6, 12 and 24 mo, and remains significant overall. These results are robust as the failsafe numbers (i.e. number of medium sized non significant studies needed to reduce the effect size to a non significant value) are relatively high (8, 16 and 33 for 6 mo, 12 mo and overall bleeding rates, respectively). This may be due to the fact that all the banding ligation studies were in favour of combination therapy concerning rebleeding rates[12,13]. Indeed, the subgroup analysis including banding ligation studies only was strongly in favour of combined treatment concerning bleeding rates ($P < 0.0001$). However, we are aware that the number of studies using banding ligation included in this meta-analysis is relatively low ($n = 3$) compared to the number of studies using sclerotherapy ($n = 14$). This difference is due to the fact that sclerotherapy is an older and more widespread technique compared to banding ligation, and that the highly significant results in favour of combined therapy in the three banding ligation studies would not have been conducive to the completion of further similar studies. Kumar *et al*[28] suggested that the pooling of sclerotherapy and banding ligation studies as in the meta-analysis of Gonzalez *et al*[51] would lead to inferior results for endoscopic therapy as sclerotherapy is known to be inferior to banding ligation for the treatment of variceal bleeding. We therefore decided to perform a subgroup analysis including only banding ligation trials as suggested by a recent editorial[52], which showed a significant decrease in rebleeding rates for banding ligation associated with β-blockers. Although only three studies were concerned, this result is relatively robust as four additional medium-sized non significant studies would be needed to render the result insignificant.

As with bleeding rates, a significant difference in mortality in favour of the combination β-blocker and endoscopic therapy group appeared when sclerotherapy and banding ligation studies were pooled. A significant difference was present overall and at 24 mo, perhaps due to the fact that the numbers of deaths at 6 and 12 mo were too low to reveal any significant statistical difference [at 6 mo: 39/309 (12.6%) for the endoscopic therapy group and 33/319 (10.3%) for the combined therapy group; at 12 mo 33/246 (13.4%) and 30/259 (11.6%), respectively]. It could be hypothesised that this decrease in mortality may be due to reduced bleeding rates, but one must keep in mind that although mortality rates are a standard end-point of all clinical trials and meta-analyses concerning variceal bleeding, it is difficult to attribute decreased mortality to decreased bleeding rates in a cirrhotic population prone to various life-threatening complications. β-blockers could have a beneficial effect on survival independent of their preventative effect on variceal bleeding as they act globally by reducing portal hypertension, and it has been suggested that they may reduce the frequency of complications of cirrhosis such as ascites, hepato-renal syndrome, portal hypertensive gastropathy[53,54] and spontaneous bacterial peritonitis[55,56]. However, the results concerning decreased mortality in the combination endoscopic and β-blocker therapy group must be interpreted with caution as the fail-safe number is relatively low: only two additional medium-sized non significant studies would be necessary in order to render the results insignificant. Additional studies with long term follow-up, preferably using banding ligation, are necessary in order to confirm these results.

Three meta-analyses have been previously published concerning combination therapy. The meta-analyses of Gonzalez *et al*[51] and Ravipati *et al*[57] compared combination treatment with endoscopic treatment (sclerotherapy or banding ligation) alone, and found a significant difference in rebleeding rates in favour of combination therapy, whereas results were non-significant for mortality. The meta-analysis of Ravipati *et al*[57] included 11 sclerotherapy trials, compared to 14 trials (148 additional patients) in our meta-analysis. Cheung *et al*[58] performed a small meta-analysis including four trials comparing banding ligation with banding ligation plus β-blockers, and did not find any significant difference for either mortality or bleeding rates. The meta-analysis of Gonzalez *et al*[51] included 23 trials. However, in contrast to our meta-analysis, these three meta-analyses[51,57,58] included studies associating β-blockers with nitrates, as well as studies in which β-blockers were introduced only at the end of variceal eradication. We decided not to include these studies for both medical and methodological reasons. Firstly, there is no proof in current literature that the association of nitrates with β-blockers is superior to β-blockers alone concerning bleeding rates and mortality. One non-blinded trial found a significantly lower bleeding rate for patients treated with nadolol plus isosorbide mononitrate[59]; however, two larger, more recent double-blinded placebo controlled trials[60,61] were unable to confirm these results, with a greater number of side effects noted in the nitrate group[60]. This association, which is poorly tolerated in certain patients, has therefore not yet been recommended by either the Baveno IV Consensus[15], or the American College of Gastroenterology[14]. Secondly, optimal combination therapy would entail introducing β-blockers at the beginning of endoscopic treatment as they would protect against bleeding recurrence before complete eradication, by reducing portal hypertension. We therefore chose not to include studies which started β-blocker treatment after eradication. However, we included all studies which started β-blockers at the beginning of eradication regardless of whether β-blocker treatment was continued after eradication or discontinued at the end of the eradication programme, as so far no studies have shown a difference in superiority between these two treatment protocols. Thirdly, including studies in which β-blockers are associated with nitrates or started only after eradication may considerably increase intertrial heterogeneity due to differences in treatment protocols. This methodological aspect has been an object of criticism in a recent editorial concerning a previous meta-analysis[52]. We therefore did not include studies associating β-blockers

with nitrates. Our stricter study inclusion criteria may lead to more precise conclusions concerning optimal treatment modalities.

The results of clinical trials and meta-analyses especially should always be interpreted with caution. A recent trial comparing banding ligation alone with banding ligation associated with drug therapy[28] did not find any significant difference in bleeding rates or mortality. However, in this trial drug therapy consisted of β-blockers associated with nitrates, as opposed to β-blockers alone, as in the trials included in the present meta-analysis. Our meta-analysis was limited by variations in quality of the studies included, and by lack of data concerning studies published as abstracts or letters, for which trial and patient characteristics were incomplete. The time over which data was included in this study was relatively large (1986-2005), which may contribute to inter-trial heterogeneity as improvements in patient care over time would affect bleeding and mortality outcomes. Data was insufficient to determine the effect of the date of trials on outcome. Likewise, subgroup analyses according to criteria such as Child's class, origin of cirrhosis or variceal size were not performed due to incomplete data. We nonetheless decided to include abstracts and letters in our meta-analysis in order to ensure optimal data analysis (246 additional patients). Adverse events were difficult to evaluate as the quality of reporting varied and most trials did not distinguish serious from non serious events. Nine studies did not perform intention-to-treat analysis whereas 2 did not state whether this was the case or not, which may lead to overestimation of treatment effect. Calculation of outcome measures at different time points reduced heterogeneity due to varying follow-up durations, but these results were limited by the fact that not all studies expressed outcome measures according to follow-up time. Ideally, a meta-analysis would include individual patient data with updated follow-up, but in our case these data were not available and we therefore had to rely on group data provided by each study. Nevertheless, we aimed to ensure the robustness of our meta-analysis results by calculating the failsafe number for each significant result, which was not estimated in previous meta-analyses[51,57,58].

In conclusion, we show that combination endoscopic and β-blocker therapy is more effective than endoscopic therapy alone in secondary prophylaxis of variceal bleeding, with a significant decrease in bleeding rates and mortality. With banding ligation becoming more and more widespread, we believe that combination β-blocker and banding ligation therapy should be recommended as first line prophylactic treatment in cirrhotic patients who have already bled from oesophageal varices.

COMMENTS

Background

Upper gastro-intestinal bleeding from ruptured oesophageal varices is a frequent and serious complication of cirrhosis. It has been estimated that 70% of patients who survive a first variceal bleeding episode subsequently rebleed. Secondary prophylaxis of variceal bleeding is therefore crucial in the management of these patients.

Research frontiers

Both β-blockers and endoscopic treatment (alone or in combination) have been used to prevent variceal rebleeding. However, there is currently no consensus as to which treatments are the most effective in secondary prophylaxis of variceal bleeding.

Innovations and breakthroughs

This meta-analysis shows for the first time that mortality is significantly decreased when β-blockers are associated with either sclerotherapy or banding ligation compared to endoscopic therapy alone. Bleeding rates are also decreased with combination β-blocker and endoscopic therapy.

Applications

The results suggest that combination β-blocker and endoscopic therapy should be recommended as the first line treatment for the secondary prophylaxis of oesophageal variceal bleeding. Additional studies with long-term follow-up are needed to confirm the results concerning mortality.

Peer review

This topic is important and there is still a considerable difference of opinion in the literature. The authors have done an exacting job of describing the conditions of their meta-analysis so that the readers can easily put the findings into the context of their own practice.

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BRIEF ARTICLE

Hepatocellular carcinoma treated with transarterial chemoembolization: Dynamic perfusion-CT in the assessment of residual tumor

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Abstract

AIM: To asses the value of computed tomography (CT)-perfusion in the detection of residual hepatocellular carcinoma (HCC) vascularization after transarterial chemoembolization (TACE).

METHODS: Thirty-two consecutive patients were prospectively included in this study. All patients had liver cirrhosis and a confirmed HCC lesion which was treated with TACE. One month after treatment, perfusion measurements of treated lesions were carried out. The CT-perfusion (CT-p) protocol was performed with 16 slice multidetector computed tomography which included the following parameters: 8 dynamic slices/scan per 40 scans after iv injection of 50 mL of iodinated contrast (350 mg/mL) at a flow rate of 6 mL/s. Treated lesions were evaluated using dedicated perfusion software, which generated a quantitative colour map of perfusion. The following parameters were considered: hepatic perfusion (HP), arterial perfusion (AP), blood volume (BV), hepatic perfusion index (HPI), and time to peak (TTP). Perfusion parameters were described with quartile values of their distribution and statistically analyzed.

RESULTS: Perfusion parameters of the treated lesions could be quantitatively assessed using CT-p analysis. The presence of residual tumor tissue was observed in 13 of the 32 patients. The values of the perfusion parameters measured within the relapse tissue were: HP (mL/100 g per minute): median = 44.4 (1^{st}qt = 31.3, 3^{rd}qt = 55.8); BV (mL/100 g): median = 18.7 (1^{st}qt = 11.5, 3^{rd}qt = 22.5); AP (mL/min): median = 39.0 (1^{st}qt = 36.5, 3^{rd}qt = 61.3); HPI (%): median = 34.0 (1^{st}qt = 30.4, 3^{rd}qt = 38.9); TTP (s): median = 17.3 (1^{st}qt = 15.8, 3^{rd}qt = 26.5). With the use of the univariate paired Wilcoxon signed rank test, HP, AP and HPI were shown to be significantly higher ($P < 0.001$) in the relapse site than in the primary lesion. The BV and TTP parameters showed a tendency to be greater and lower, respectively, in the relapse site than in the primary lesion.

CONCLUSION: In patients with HCC treated with TACE, CT-p provides measurement of flow parameters related to residual arterial structures in viable tumor, thus helping in the assessment of therapeutic response.

Key words: Computed tomography-perfusion; Functional-computed tomography; Hepatocellular carcinoma; Trans-arterial chemoembolization; Tumour neo-angiogenesis

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INTRODUCTION

The prognosis of patients with untreated hepatocellular carcinoma (HCC) is poor, with a median survival of less than 6 mo after diagnosis. Although surgery remains the only hope for cure, very few patients (10%-15%) are candidates[1]. For large HCCs (> 3 cm), transarterial chemoembolization (TACE) remains the sole approach to inhibit cancer growth in most patients, and has been used extensively to treat unresectable HCCs[2]. The rationale for chemoembolization stems from the observation that unlike normal liver tissue, HCC receives most of its blood supply from the hepatic artery, therefore, it is logical to use the hepatic artery as a means to target the tumor while preserving normal liver[1]. The goal of TACE is to determine tumor necrosis and control tumor growth while preserving as much functional liver tissue as possible[1], therefore, the early detection of residual or recurrent tumor after TACE is a critical point and can facilitate successful retreatment at an early stage. Usually the presence of residual viable tumor or recurrence after TACE is assessed using multiphasic multidetector computed tomography (MDCT) scanning, but this technique can be affected by artefacts produced by high concentrations of lipiodol, making it difficult to evaluate the characteristics of the lesion. Response may be better assessed by noting alterations in vascular tumor perfusion rather than size, and therefore functional measurements may be an appropriate method to assess tumor response.

Perfusion computed tomography (CT) is a technique that enables depiction of tumor vascular physiology, being, also, non-invasive and fast; perfusion-CT can be repeated sequentially to assess temporal changes in tumor blood flow, which is of clinical importance for monitoring tumor response to antiangiogenic agents and other treatments[3].

The utility of hepatic perfusion characterization relies on the resolution of each component of its dual blood supply-the portal vein and the hepatic artery-because contributions from each are altered predictably in many diseases and could be used in monitoring therapeutic effectiveness[4]. TACE is a liver-directed therapy that takes advantage of the relatively selective vascularization of hepatic arterial tumors. HCCs can derive approximately 80% to 85% of their blood supply from the hepatic artery, whereas the portal vein as well as the hepatic artery supplies the normal hepatic parenchyma[4].

Quantitative measurement of hepatic perfusion has the potential to provide important information about tumor vascularization, useful in the assessment and management of various liver diseases and in the determination of their treatment outcome[5].

The purpose of this study was to determine the value of functional CT with perfusion imaging in the quantitative assessment of blood flow changes related to the effects of TACE treatment in patients with HCC lesions.

MATERIALS AND METHODS

Patients

According to the Barcelona staging classification[6] TACE was performed in 35 consecutive cirrhotic patients with otherwise untreatable HCC, and included 28 men and 7 women (mean age, 65 years; range, 45-76 years). The cause of cirrhosis was hepatitis infection in 24 patients (hepatitis B in 7 patients, hepatitis C in 17 patients), alcohol related in 4 patients, and both hepatitis C and alcohol related in 7 patients.

To be included in the study, patients had to meet the following criteria: (1) be older than 18 years; (2) Child-Pugh class A or B; (3) have focal or multifocal HCC; and (4) not have any contraindications to CT imaging. Excluded from the study were patients who had: (1) a life expectancy of less than 6 mo; (2) Child-Pugh class C; (3) uncorrectable coagulopathy (international normalized ratio > 1.5); (4) thrombosis of the main portal vein branches (portal venous thrombosis was excluded prior to TACE by means of Doppler US); (5) a total bilirubin level higher than 4.0 mg/dL; (6) a serum creatinine level higher than 1.7 mg/dL; and (7) thrombocytopenia (platelet count < 50000/μL). The diagnosis of HCC was established with FNAB or on the basis of the presence of a tumor larger than 2 cm in diameter with typical imaging findings[6] in the setting of cirrhosis.

Before being enrolled, all subjects gave their informed consent after the nature of the procedure had been fully explained, in accordance with the regulations of the institutional review board that approved our study.

According to the current literature[7-9], CT is commonly used as the standard imaging technique for evaluating the therapeutic response in patients with HCC after TACE[8], and was performed 4 wk after TACE treatment, in order to assess the intralesional deposition of iodized oil and evaluate the presence of the residual viable portion of the treated lesion[6,8].

All patients underwent CT-perfusion 1 d after the MDCT study and subsequently a selective conventional

DSA study to correlate the angiographic findings with those of the CT-perfusion examination.

TACE treatment

After initial mapping, visceral conventional DSA with a selective 5-F visceral catheter [either: Simmons (Cook, Bloomington, IN, USA) or Cobra (Terumo Medical, Somerset, NJ, USA) catheters were used], mesenteric arteriography was performed to check for presence of the right hepatic artery. Indirect portography was performed next to outline the portal circulation in the venous phase. Depending on the size, location, and arterial supply of the tumor and its satellites, the tip of the catheter was advanced further into segmental arteries for selective embolization, typically we coaxially inserted a 2.8-F microcatheter (Renegade Hi-Flow; Boston Scientific, Natick, MA, USA) over a 0.016-inch-diameter guidewire (Headliner; Terumo, Tokyo, Japan) to super select the hepatic lobar or segmental hepatic artery supplying the targeted tumor. We performed conventional DSA following an injection of Iobitridol (Xenetix 350; Guerbet, Aulnay, France).

Once the operator selected the final catheter position for TACE, intraarterial chemotherapy was performed by injection of 10-12 mL of iodized oil (Lipiodol Ultra Fluide; Laboratoire Guerbet, Roissy, France) mixed with an emulsion of 40 mg of doxorubicin hydrochloride into the hepatic artery. Embolization was performed by means of a mixture of Iobitridol (Xenetix 350; Guerbet, Aulnay, France) and 1-mm-diameter absorbable gelatin sponge particles (Spongostan; Ferrosan, Søborg, Denmark).

Under direct radiographic monitoring, we injected the solution (mean dose, 9.4 mL; range, 2-18 mL) until some slowing of antegrade blood flow to the targeted tumor was identified with conventional fluoroscopy. The amount of injected chemoembolic material was chosen at the discretion of the attending interventional radiologist. After the TACE procedure, the patients recovered with 20-24 h bed rest.

Perfusion CT technique

Perfusion-CT was performed using a multi-detector 16-slice CT scanner (Brilliance, Philips Medical Systems, Eindhoven, The Netherlands). For selection of the appropriate transverse level, an unenhanced CT scan of the liver, performed during a single breath hold, was obtained before initiation of perfusion CT scanning. The perfusion study of the selected area (maximal tumor diameter) was performed in a single breath hold at the end of expiration. Single location (2.4 cm width) cine CT scanning (40 scans; eight slices/scan) was obtained using the following parameters: 120 kV, 120 mA, 512 × 512 matrix, 3-mm slice thickness, and 1-s scan time. The radiation dose delivered to each patient with our CT perfusion technique was 8 mSv. Oxygen inhalation (4 mL/min) was given to help the patients hold their breath during the examination. Scanning was initiated after a 7-s delay from the start of the intravenous bolus injection of 50 mL of non-ionic iodinated contrast agent (Xenetix 350; Guerbet, Aulnay, France) at a flow rate of 6 mL/s, using an 18-gauge catheter positioned in the antecubital vein. Dynamic CT images were acquired for a total duration of 40 s.

To avoid motion artefacts, patients were clearly informed of a possible flushing sensation commonly associated with a rapid bolus of iodinated contrast agent. Moreover, as image quality may be degraded in patients breathing deeply during acquisition resulting in inaccurate perfusion parameters values, a band compressing the abdomen that limited breath-related liver excursions was used.

Image analysis and quantification of perfusion parameters

The functional computed tomographic images were then transferred to an image workstation, and the data were analyzed using dedicated perfusion software (Philips Brilliance Workspace 2.0; Philips Medical Systems), which generated a quantitative map of liver perfusion displayed on the monitor using a colour scale. The parametric map images were created using the highest spatial resolution pixel-by-pixel calculation technique. Perfusion was assessed by the dedicated computed tomographic software based on the maximum slope model: perfusion was therefore calculated as the average slope of the tissue enhancement divided by the peak enhancement in the aorta, as described initially by Miles and Griffiths[4,10].

The region of interest (ROI) was hand drawn in a selected portion of the treated lesion where a hypervascular area had been detected on the colour map (Figures 1 and 2); another ROI was positioned within the intralesional deposition of iodized oil (Figures 1 and 2); further ROIs were also placed in definite areas of the surrounding cirrhotic liver parenchyma (Figures 1 and 2). In those patients in whom the presence of a focal hypervascular area, suspected as residual HCC, had not been seen at the CT-perfusion study, ROI was positioned only on the site of deposition of iodized oil and in the surrounding liver parenchyma, and the perfusion parameters were calculated. The resulting temporal changes in contrast enhancement were then analyzed to quantify a range of parameters that reflected the functional status of tissue perfusion.

The absolute values of the following five perfusion parameters were calculated for treated HCC, and for cirrhotic liver parenchyma: (1) Hepatic perfusion (HP), mL/100 g per minute), which is the blood flow per unit volume/mass of tissue per minute; (2) Blood volume (BV, mL/100 g), which is the blood volume contained in 100 g of tissue; (3) Arterial perfusion (AP, mL/min), which is the arterial fractional blood flow; (4) Hepatic perfusion index (HPI, %), which represents, of the total blood liver flow [arterial perfusion (AP) + portal perfusion (PP)], the percentage of arterial origin (AP/AP + PP); and (5) Time to peak (TTP, s) which is the time to reach the maximum value of contrast material concentration.

In only three of 35 patients, the perfusion study was not performed due to the fact that the tumor and portal trunk, or main portal branches, were not on the same transverse plane.

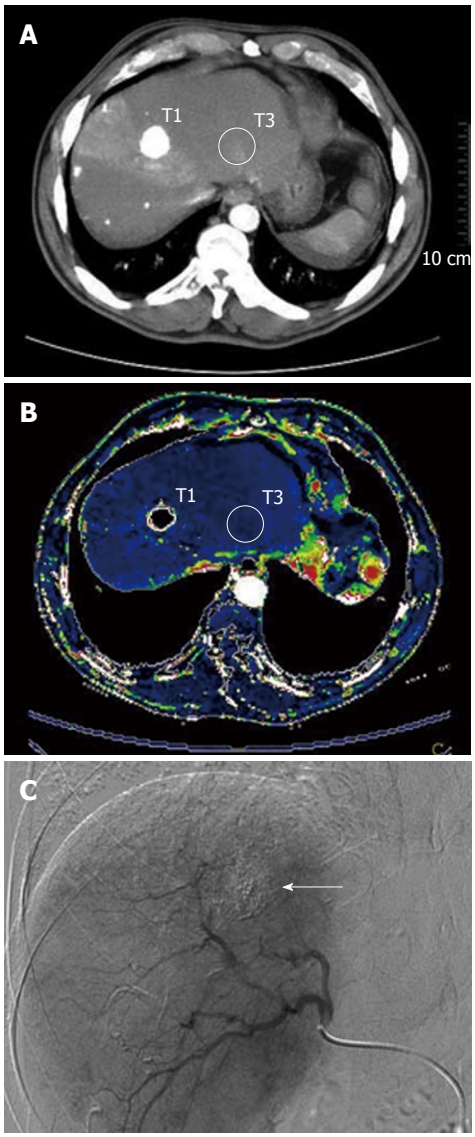

Figure 1 A 69-year-old man with Child A liver cirrhosis and 12 mm lesion of hepatocellular carcinoma, treated with transarterial chemoembolization, in the seventh segment of the liver that underwent computed tomography-perfusion study. A: Multiphasic multidetector computed tomography scan image shows two hand drawn regions of interest (ROIs): T1 positioned on the successfully treated tumor contouring the treated area; T3 positioned on the surrounding liver parenchyma avoiding vessel structures, in order to obtain the quantitative perfusion data of the regions; B: Functional arterial perfusion colour map shows an homogenous distribution of perfusion area, with decreased perfusion range of colors compared with the background liver parenchyma; ROIs were positioned at the same level, on the successfully treated tumor (T1) and on the surrounding liver parenchyma (T3); C: Post chemoembolization digital fluoroscopic image obtained in the same patient shows excellent distribution of the lipiodol-chemotherapy mixture (arrow).

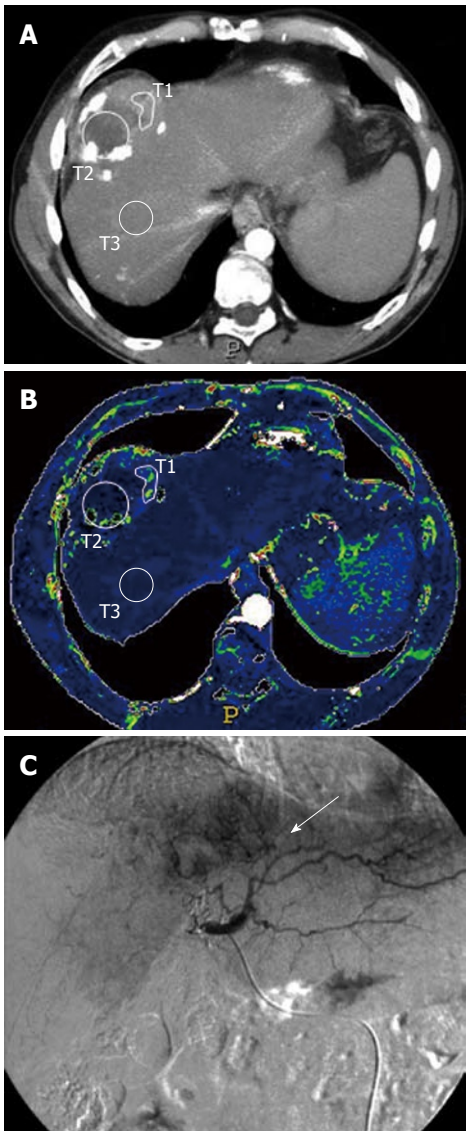

Figure 2 A 57-year-old man with Child B liver cirrhosis and 42 mm lesion of hepatocellular carcinoma in the fourth segment of the liver that underwent computed tomography-perfusion study. A: Transverse data raw multiphasic multidetector computed tomography scan image shows regions of interest (ROIs) positioned on the periphery of lesion (T2), and on the primary lesion without relapse (T1) and on the surrounding liver parenchyma (T3) avoiding vessel structures, in order to obtain the quantitative perfusion data of the regions; B: Functional arterial perfusion colour map shows that the distribution of perfusion in the treated lesion is heterogeneous, with a different range of colours of residual disease (T1) compared with primary lesion without relapse (T2) that reveal the unsuccessful treatment of transarterial chemoembolization; ROIs were also positioned at the same level in surrounding liver parenchyma (T3); C: Post chemoembolization digital fluoroscopic image obtained in the same patient shows disomogeneous distribution of the iodized lipiodol-chemotherapy mixture, with presence of hypervascular region (arrow) at the periphery of treated lesion, demonstrating the viable portion of tumour.

Statistical analysis of perfusion parameters

The perfusion parameters HP, BV, AP, HPI, TTP were described by the quartile values of their distribution and calculated: for the ROI in the area of iodized oil deposition (T1) in all patients (with or without residual disease); for the ROI in the hypervascular area (T2) at the colour map in 13 patients who presented residual neoplastic disease; for the ROI placed in the surrounding liver parenchyma (T3) in all patients.

The distributions of the differences in the parameters between the residual disease, deposition of iodized oil and surrounding cirrhotic liver tissue were graphically represented by means of box-plot representations (Figure 3).

The univariate unpaired Wilcoxon signed rank test was used to assess whether there were differential expressions of each single perfusion parameter in the area of deposition of iodized oil between both groups of pa-

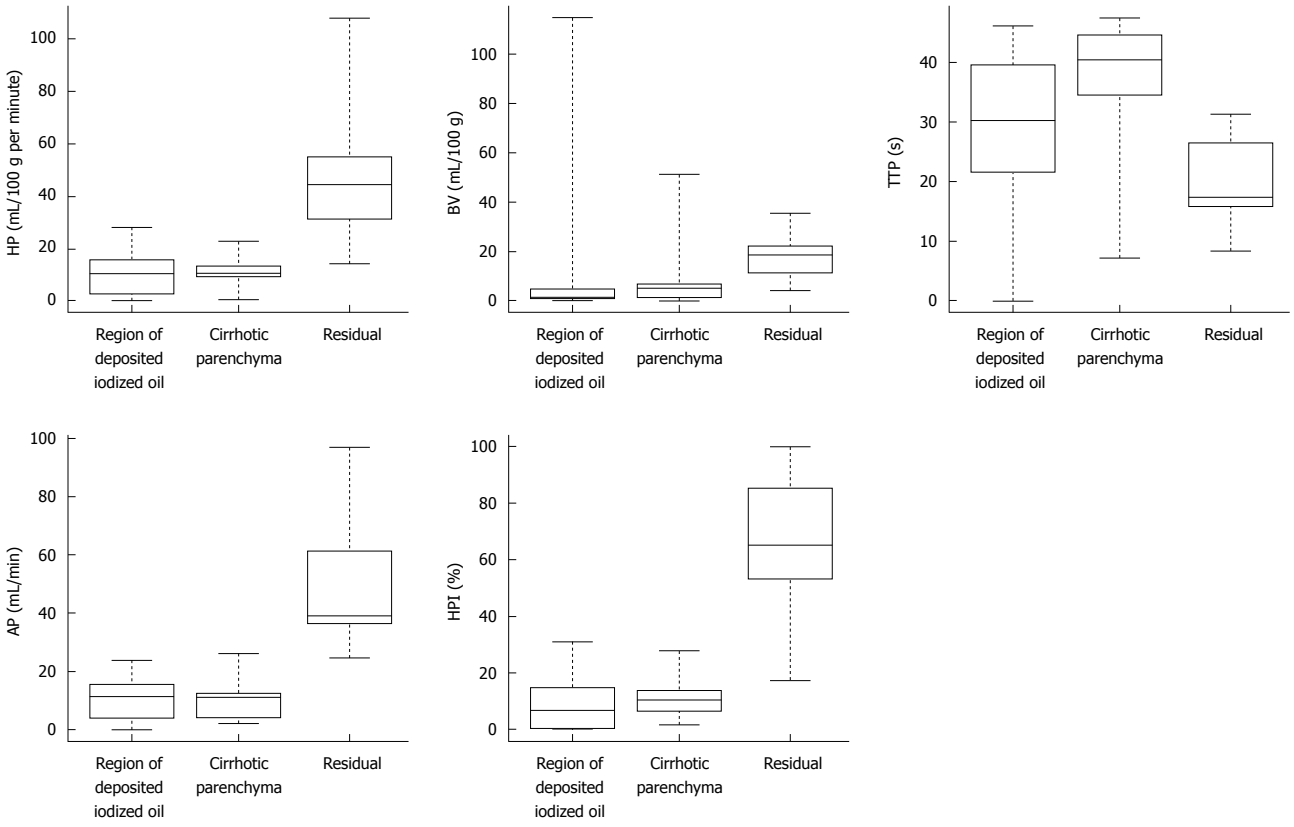

Figure 3 Box plots of the differences in perfusion parameters (hepatic perfusion, tissue blood volume, hepatic perfusion index, arterial perfusion, and time to peak). The lower boundary of the boxes indicates the 25th percentile, line within the boxes marks the median, and the higher boundary of the boxes indicates the 75th percentile. Error bars below and above the boxes indicate minimum and maximum values. HP: Hepatic perfusion; BV: Blood volume; HPI: Hepatic perfusion index; AP: Arterial perfusion; TTP: Time to peak.

tients (those who did and did not present residual HCC). While, the paired version of the Wilcoxon test was used to assess whether, in patients who had residual disease, there were differential expressions of the perfusion parameters between the area of deposition of iodized oil and the residual site.

Bonferroni correction was used to take multiple comparisons into account, considering *P*-values less or equal to 0.05/10 were indicative of a statistically significant difference[11].

In the comparison between the area of deposition of iodized oil and the residual site for patients who had residual disease, the alternative hypothesis postulated a greater value for, HP, BV, AP and HPI in the residual disease than in the area of deposition of iodized oil or cirrhotic parenchyma, due to the presence of residual arterial vascular structures. For TTP, the alternative hypothesis postulated a lower value for the residual disease site than the primary lesion, due to a possible increase in the arterial flow within the tumor.

The statistical analysis was performed using the R statistical package (http://cran.r-project.org).

RESULTS

A total of 32 lesions (mean size: 4.1 ± 1.6 cm), located on both hepatic lobes were evaluated with the CT-Perfusion (CTp) technique.

For all patients, the descriptive analysis of perfusion parameters measured within the treated tumor tissue with deposition of iodized oil (T1) showed the following results.

HP (mL/100 g per minute): median = 10.5 (1^{st}qt = 9.3, 3^{rd}qt = 13.7); BV (mL/100 g): median = 5.2 (1^{st}qt = 1.3, 3^{rd}qt = 6.7); AP (mL/min): median = 11.2 (1^{st}qt = 4.0, 3^{rd}qt = 12.5); HPI (%): median = 10.7 (1^{st}qt = 6.4, 3^{rd}qt = 13.7); TTP (s): median = 40.3 (1^{st}qt = 34.6, 3^{rd}qt = 44.6) (Table 1, Figure 3).

In the 13 patients who presented residual disease, the same descriptive analysis of perfusion parameters was performed within the residual tumor tissue (T2) and showed the following results: HP (mL/100 g per minute): median = 44.4 (1^{st}qt = 31.3, 3^{rd}qt = 55.8); BV (mL/100 g): median = 18.7 (1^{st}qt = 11.5, 3^{rd}qt = 22.5); AP (mL/min): median = 39.0 (1^{st}qt = 36.5, 3^{rd}qt = 61.3); HPI (%): median = 65.0 (1^{st}qt = 53.2, 3^{rd}qt = 85.0); TTP (s): median = 17.3 (1^{st}qt = 15.8, 3^{rd}qt = 26.5) (Table 1, Figure 3).

The corresponding perfusion values calculated also in the cirrhotic liver parenchyma surrounding the treated HCC lesion were: HP (mL/100 g per minute): median = 10.4 (1^{st}qt = 9.3, 3^{rd}qt = 13.2); BV (mL/100 g): median = 11.7 (1^{st}qt = 9.5, 3^{rd}qt = 11.9); HPI (%): median = 16.4 (1^{st}qt = 13.8, 3^{rd}qt =18.3); AP (mL/min): median = 10.4 (1^{st}qt = 9.5, 3^{rd}qt = 11.9); TTP (s): median = 44.6, (1^{st}qt = 40.3, 3^{rd}qt = 51.8) (Table 1, Figure 3).

Table 1 Descriptive characteristics of analyzed perfusion parameters

Parameter	Primary treated lesion site in region of deposited iodized oil			Background cirrhotic liver parenchyma			Residual hepatocellular carcinoma		
	Median	1st qt	3rd qt	Median	1st qt	3rd qt	Median	1st qt	3rd qt
HP (mL/100 g per minute)	10.5	9.3	13.7	10.4	9.3	13.2	44.4	31.3	55.8
BV (mL/100 g)	5.2	1.3	6.7	11.7	9.5	11.9	18.7	11.5	22.5
HPI (%)	10.7	0.3	14.9	16.4	13.8	18.3	65	53.2	85.0
AP (mL/s)	11.2	4	12.5	10.4	9.5	11.9	39	36.5	61.3
TTP (s)	40.3	34.6	44.6	44.6	40.3	51.8	17.3	15.8	26.5

HP: Hepatic perfusion; BV: Blood volume; HPI: Hepatic perfusion index; AP: Arterial perfusion; TTP: Time to peak.

With the use of the univariate paired Wilcoxon signed rank test, HP, AP and HPI were shown to be significantly higher ($P < 0.001$) in the site of residual disease than in the region with iodized oil deposition (Figure 3). The BV parameter showed a tendency to be greater in the residual site than in the region of iodized oil deposition, but significance according to the Bonferroni correction was not reached ($P = 0.03$) (Figure 3). The TTP values showed a tendency to be lower in the site of residual disease than in the region of iodized oil deposition, but again significance according to the Bonferroni correction was not reached $P = 0.01$) (Figure 3).

Use of the univariate unpaired Wilcoxon signed rank test showed that no significant differences were found for each parameter, calculated in the region of the lesion with deposition of iodized oil, between the two groups of patients with or without relapse of HCC (Figure 3).

In our series no significant correlation was found between CT perfusion parameters in cirrhotic parenchyma and in the successfully treated lesion, although in lesions with deposition of iodized oil mean BV value was moderately lower than those in the surrounding cirrhotic parenchyma.

The survival rate at 6, 12 and 18 mo was 94% (30/32), 82% (26/32) and 72% (23/32), respectively.

DISCUSSION

TACE is the most widely used therapy in patients with HCC who are considered unsuitable candidates for surgery, and should be considered not only the best possible therapeutic approach in patients with advanced HCC but also an appropriate step before surgical resection or liver transplantation[12].

The mixture of chemotherapeutic agents and iodized oil is almost completely retained in neoplastic nodules and can remain in HCC tissue for a long time. Subsequent mechanical embolization of the artery feeding the neoplasm causes ischemic damage to the tumor and prolongs the duration of the effects of chemotherapeutic agents[13].

For a definite assessment of the therapeutic efficacy of interventional procedures, histological examination using percutaneous needle biopsy may be the most definite assessment of the therapeutic efficacy of interventional therapy. However, it is an invasive procedure, and the specimen retrieved does not always represent the entire lesion owing to sampling errors.

Similar to the findings in most previously reported investigations[7,8], the presence of residual viable tumor or recurrence after TACE is assessed using multiphasic MDCT scanning, which represents the standard imaging technique for monitoring the effectiveness of TACE.

Generally, HCC tends to recur at the site adjacent to the original tumor, however, after TACE treatment it can be difficult to evaluate contrast enhancement in a tumor with partial retention of iodized oil on contrast-enhanced CT due to beam hardening artefacts produced by the high attenuation of iodized oil[12].

In general, the viable portion of malignant tumors exhibits increased perfusion, and in the primary tumors, therefore, the radiologic estimation of perfusion in liver tumors is useful for both diagnosis and selection of the therapeutic strategy[14].

After TACE, tumor response may be better assessed by alterations in vascular perfusion rather than tumor size, and functional measurements may therefore be more appropriate[15].

In this regard, perfusion-CT could be considered suitable for monitoring tumor response after treatment.

Perfusion CT techniques typically require a baseline image acquisition without contrast enhancement followed by a series of images acquired over time after an intravenous bolus of conventional iodinated contrast material. The resulting temporal changes in contrast enhancement are subsequently analyzed to quantify a range of parameters that reflect the functional status of the vascular system[16].

In the current study, we investigated the value of several tissue perfusion parameters obtained by functional CT, with the perfusion technique, for the quantitative assessment of HCC-related residual vascularization after TACE treatment.

Our results show that the values of the perfusion parameters calculated were significantly different in residual tumor compared to the area of iodized oil deposition, in particular, HP, AP, BV and HPI were found to be higher ($P < 0.001$) in HCC than in the surrounding cirrhotic liver tissue, due to tumor-associated neovascularization, whereas TTP values were lower in areas of HCC relapse. In the assessment of residual neovascularization related to tumor regrowth, AP - which represents the arterial fractional blood flow - may be considered the most rel-

evant perfusion parameter, as it can specifically demonstrate the presence of new arterial blood vessels in partial or incomplete treated lesions. Also, the higher HPI values could explain the reduction of portal perfusion inflow in residual HCC disease, with a simultaneous increase in arterial fractional inflow.

Findings in the current work are also in line with those recently reported in experimental studies where perfusion CT was used for assessing tumor response to treatment by evaluating perfusion changes[17-19]. Those studies validated the idea that functional CT can provide quantification of residual viable tumor perfusion. In an animal model, Kan *et al*[17] found that functional CT enabled accurate quantification of changes in liver tumor perfusion during and after an embolization procedure thus helping optimize therapeutic outcomes. In another study[18], the same researchers reported on the ability of functional CT to assess changes in liver tumor perfusion in response to antiangiogenic treatment.

In our study, we found that the zone of increased perfusion appeared as a hypervascular area on the colour map, representing an area in which the arterial blood continued to sustain tumor growth.

Our findings are in line with a previous study by Chen *et al*[19] who correlated the changes of CT-perfusion parameters to different responses of tumors to TACE, supporting the idea that CT-perfusion could be used in the assessment of TACE efficacy, by evaluating quantitative parameters before and after chemoembolization.

Our data confirmed the recent experimental work of Sabir *et al*[15], who demonstrated in a murine xenograft tumor model receiving antiangiogenic therapy, that perfusion MDCT was able to identify changes in tumor blood flow, and therefore indicate the early reversal of tumor responsiveness to antiangiogenetic therapy. This also demonstrated, therefore, that perfusion MDCT is able to identify focal areas of new tumor perfusion before clinically measurable changes in tumor size.

Our study findings are consistent with those of Li *et al*[20] who demonstrated that CT perfusion parameters can be reliable indicators for evaluating tumor necrosis and angiogenesis, relating the dynamic contrast-enhanced measures with outcome of histopathologic (microvessel density) studies in lung carcinoma. Evaluating the CT perfusion parameters of the necrotic lung carcinomas, they found that necrotic tumors exhibited significantly lower perfusion.

The main limitation of the present study is related to the fact that a liver tissue section of only 2.4 cm in thickness could be examined in each patient. This limitation, however, could be overcome by the use of multi-detector CT scanners enabling the acquisition of 64 or more sections which can produce isotropic spatial resolution, while simultaneously delivering exceptional temporal resolution with excellent z-axis coverage (4-8 cm)[21]. A further limitation was that CT perfusion parameters were not compared with established markers of tumor vascularity, such as histologic examination, which is the most reliable method for assessing therapeutic efficacy of the TACE procedure. Such a method, however, is invasive and the specimen retrieved does not always represent the entire lesion owing to sampling errors[6].

In conclusion, our preliminary study in humans has shown that perfusion CT may be helpful in monitoring the outcome of TACE in cirrhotic patients with HCC. We believe that perfusion-CT provides non-invasive, reliable information on residual tumor vascularization and could be part of the current CT protocols aimed at following-up such patients.

COMMENTS

Background

Transcatheter arterial chemoembolization (TACE) is the most widely used therapy in patients with hepatocellular carcinoma (HCC) who are considered unsuitable candidates for surgery. Chemoembolization involves delivery of chemotherapy combined with arterial embolization to destroy tumor cells. Such a treatment makes use of the hypervascular nature of HCC: antineoplastic agents are directly injected into the hepatic artery allowing high intratumoral concentrations of drugs and thereby reducing systemic side effects. After TACE, tumor response may be better assessed by alterations in vascular perfusion rather than tumor size, and functional measurements may therefore be more appropriate. Thus, quantitative measurement of hepatic perfusion has the potential to provide important information in the assessment and management of HCC lesions and in the determination of their outcome.

Research frontiers

Perfusion computed tomography is a recent technique that allows depiction of tumor vascular physiology and has the ability to detect regional and global alterations in organ blood flow, in addition to being non-invasive and fast; perfusion computed tomography (CT) can be repeated sequentially to assess temporal changes in tumor blood flow, which is of clinical importance for monitoring tumor response to antiangiogenic agents and other treatments like TACE.

Innovations and breakthroughs

This new technique may better define the assessment of therapeutic efficacy of interventional procedures without using histological examination, evaluate tumor progression and monitor response to treatment providing a quantitative measurement of flow parameters related to residual arterial structures in viable tumor. As it is a relatively simple imaging technique, perfusion CT could be integrated into the current computed tomographic protocols, providing an *in vivo* marker of tumor-related angiogenesis.

Applications

This preliminary study in humans has shown that perfusion CT may be used to successfully monitor therapeutic response after TACE in cirrhotic patients with HCC lesions. In addition, the follow-up protocol along with conventional multi-detector computed tomography may include a CT-perfusion protocol in order to better depict residual tumor after TACE treatment, by providing quantitative information about tumor vascularization.

Peer review

The authors assess the value of CT-perfusion technique in detection of residual HCC vascularization after TACE. It's a nice imaging clinical study to follow up patients with HCC.

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BRIEF ARTICLE

Immune phenotype in children with therapy-naïve remitted and relapsed Crohn's disease

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Abstract

AIM: To characterize the prevalence of subpopulations of $CD4^+$ cells along with that of major inhibitor or stimulator cell types in therapy-naïve childhood Crohn's disease (CD) and to test whether abnormalities of immune phenotype are normalized with the improvement of clinical signs and symptoms of disease.

METHODS: We enrolled 26 pediatric patients with CD. 14 therapy-naïve CD children; of those, 10 children remitted on conventional therapy and formed the remission group. We also tested another group of 12 children who relapsed with conventional therapy and were given infliximab; and 15 healthy children who served as controls. The prevalence of Th1 and Th2, naïve and memory, activated and regulatory T cells, along with the members of innate immunity such as natural killer (NK), NK-T, myeloid and plasmocytoid dendritic cells (DCs), monocytes and Toll-like receptor (TLR)-2 and TLR-4 expression were determined in peripheral blood samples.

RESULTS: Children with therapy-naïve CD and those in relapse showed a decrease in Th1 cell prevalence. Simultaneously, an increased prevalence of memory and activated lymphocytes along with that of DCs and monocytes was observed. In addition, the ratio of myeloid /plasmocytoid DCs and the prevalence of TLR-2 or TLR-4 positive DCs and monocytes were also higher in therapy-naïve CD than in controls. The majority of alterations diminished in remitted CD irrespective of whether remission was obtained by conventional or biological therapy.

CONCLUSION: The finding that immune phenotype is normalized in remission suggests a link between immune phenotype and disease activity in childhood CD. Our observations support the involvement of members of the adaptive and innate immune systems in childhood CD.

Key words: Crohn's disease; Dendritic cell; Infliximab; Lymphocyte; Monocyte; Regulatory T cell; Relapse; Remission; Therapy-naïve; Toll-like receptor

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INTRODUCTION

Crohn's disease (CD) is a chronic gastrointestinal disease characterized by segmental inflammation of the intestinal mucosa associated with a dysregulated action of the mucosal immune system to the otherwise innocuous luminal antigens in a genetically susceptible host. About 10%-15% of patients with CD are diagnosed before 18 years of age[1]. Certain features are unique to pediatric CD in comparison to adult onset disease such as different disease location, altered response to immunosuppressive therapy and different genetic and immune phenotype[2,3]. Different characteristics may suggest differences in the pathomechanism of CD in children compared to that in adults. Theories regarding CD pathomechanism include malfunctioning of the immune system. Indeed, several studies carried out in adult CD indicate the involvement of either adaptive or innate immunity[4-7].

In adult CD, peripheral Treg prevalence is diminished in therapy-naïve patients and in the active state of disease or relapses and is increased with therapy or in remission[8-10], while intestinal Treg prevalence is increased in active CD[9-11]. Simultaneously, the prevalence of effector T cells[12-14] and activated T cells[15-17] is increased in the periphery in active CD. The shift of T lymphocytes toward Th1 commitment in peripheral blood and biopsy specimens is a widely observed phenomenon in CD[18].

In addition, the prevalence of antigen presenting cells (APCs) including dendritic cells (DCs) decrease in remission, and even more so in relapses at the periphery, but is increased in tissues, simultaneously with an increased expression of Toll-like receptor (TLR)-2 and 4 in therapy-naïve and treated CD adults[19-21]. Monocytes/macrophages, another group of APCs, are comparable at the periphery but increased in biopsies and showed upregulation of TLR-2 and TLR-4 in the active state and in remission of CD[22-25]. Other cell types of innate immunity such as natural killer (NK) and natural killer T (NKT) cells are less prevalent in active CD[17,26-28].

There have been a limited number of studies specifically performed to investigate alterations of the immune system in children with CD. In addition, the majority of available data are on treated CD children. While in untreated CD children some alterations in adaptive immunity have been reported, such as the skewness of the Th1/Th2 ratio to Th2 (this finding is in contrast with that observed in adult CD)[29-32], recent data suggest that the disturbance of innate immune functions is also a major factor contributing to CD in children. Indeed, in untreated children the central macrophage prevalence is increased[33], and TLR-2 and 4 receptor expression is also enhanced[34]. The role of innate immune system in the pathomechanism of CD may be inversely associated with age at disease onset[2].

The immune dysregulation in CD is affected by ongoing therapy. Aminosalicylates and steroids, as well as immunosuppressive drugs used as first-line therapy in CD have strong immunomodulatory effects[35]. In addition, biological therapy including the tumor necrosis factor α inhibitor infliximab (IFX)[36] also has substantial effects on immune cell functions [many of the data are from patients with rheumatoid arthritis (RA)] as it may increase the prevalence of peripheral and central Tregs[8,37], effector and activated[38,39] and Th1 committed T cells[39,40]. IFX also decreases the prevalence of NK cells[38], DCs[41,42] and monocytes[43,44], as well as TLR-2 and TLR-4 expression in peripheral cells[45]. The immunological impact of conventional or IFX therapy on peripheral immune phenotype is, however, known exclusively for adult CD patients and has not been explored fully in CD children.

In this prospective study we aimed to explore the major cell prevalence of the adaptive and innate immune systems in therapy-naïve CD children and its alteration with the improvement of CD obtained by conventional therapy or IFX treatment.

MATERIALS AND METHODS

We enrolled the following patient groups into our study: (1) 14 therapy-naïve CD children. No drug was prescribed for these patients at the time of CD diagnosis. The diagnosis of CD was established by means of "The Porto criteria"[46]; disease activity was determined according to the Pediatric Crohn's disease activity index (PCDAI)[47]; (2) During conventional treatment [steroid, azathioprine (AZT) and 5-aminosalicylate (5-ASA)], 10 children responded forming the remission group. Clinical remission was defined as a PCDAI < 10; (3) IFX therapy (5 mg/kg IFX at weeks 0, 2, and 6) was started in 12 CD children who failed to respond to conventional therapy forming the "relapsed group". Non-responsiveness was defined as moderately increased PCDAI (PCDAI > 30) in patients under conventional therapy; and (4) Fifteen age- and gender-matched children with functional abdominal pain served as controls. All patients and controls were diagnosed, treated and followed up in the Outpatient Clinic of the First Department of Pediatrics, Semmelweis University between September 2007 and August 2009. The Institutional Ethical Committee approved our study; written parental informed consent was obtained.

The patients' clinical characteristics are shown in Table 1. Small and large bowel was involved in 11 of 14 treatment naïve CD patients, according to the literature (L3 localization, Montreal criteria[48]). Therapy-naïve CD patients and CD patients with relapse had lower body mass index than controls. Lower body weight and body mass index is a common presenting sign in pediatric patients with CD[49]. Reduced food intake, postprandial abdominal cramps, systemic release of cytokines and malabsorptive diarrhea were listed as factors responsible for this phenomenon[50].

Table 1 Clinical data and patient characteristics

	Control	Therapy-naïve (before conventional therapy)	First remission (with conventional therapy)	Relapse (before IFX therapy)	IFX therapy (before 2nd infusion)	IFX therapy (before 3rd infusion)
Clinical data						
n (boys/girls)	15 (6/9)	14 (6/8)	10 (4/6)		12 (5/7)	
Age (yr)	12 (8-16)	10 (8.5-13)	11.5 (9.5-15.5)	14 (11-16)	14.5 (11.5-16)	14.5 (12-16)
Body mass index (kg/m^2)	19.5 (16.5-22.3)	13.9 (12.5-15.8)^{d}	18.6 (14.4-19.2)^{c}	17.4 (14.1-19.5)^{a}	18.8 (14.6-21.3)	19.25 (16.1-22.1)
Body weight (percentile)	58 (35-79)	13 (4-23)^{d}	24 (19-44)^{c}	21 (7-28)^{a}	23 (15-63)	35 (10-74)
Disease duration (mo)	-	10 (8.5-13)	10.5 (8.5-15.5)	11.5 (9.25-15)	12 (9.5-15)	12 (10-15)
Activity index (PCDAI)	-	45 (39-58)	0 (0-5)^{f}	45 (25-48)^{j}	20 (7-28)^{g}	13 (2-22)^{l}
Localization (*n*, montreal criteria)	-	L1 (1), L2 (2), L3 (11)	L1 (0), L2 (2), L3 (8)		L1 (1), L2 (2), L3 (9)	
Laboratory data						
White blood cell count (g/L)	7.9 (5.7-10.1)	12.4 (9.4-14.2)^{b}	11.7 (8.4-14.4)	10 (8.2-11.2)	7.9 (5.5-10.6)	6.7 (4.5-10.4)^{e}
Platelet (g/L)	346 (288-375)	657 (451-746)^{b}	442 (308-624)	479 (396-750)^{b}	392 (293-523)	383 (308-483)^{e}
Serum iron (μmol/L)	16 (14-21)	4 (2-12)^{a}	8 (4-10)^{a}	4 (2-7)^{d}	5 (4-7)^{d}	8 (6-8)^{b,e}
Serum albumin (g/L)	45 (44-49)	37 (34-39)^{d}	42 (42-45)^{c}	40 (36-41)^{d}	41 (38-45)^{b}	42 (38-45)^{b}
C-reactive protein (mg/L)	0 (0-1)	21 (5-65)^{d}	7 (2-11)^{b,c}	27 (9-55)^{h,d}	9 (3-11)^{d}	5 (1-14)^{d,e}

$^{a}P < 0.05$, $^{b}P < 0.01$; $^{d}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{f}P < 0.01$ *vs* therapy-naïve; $^{h}P < 0.01$, $^{j}P < 0.01$ *vs* first remission; $^{e}P < 0.05$, $^{g}P < 0.05$, $^{l}P < 0.001$ *vs* relapse. IFX: Infliximab; PCDAI: Pediatric Crohn's disease activity index; L1: Small bowel; L2: Large bowel; L3: Small bowel and large bowel localization according to Montreal criteria[48].

Together with other routine blood sampling 6 mL of lithium-heparin anticoagulated blood was taken from therapy-naïve patients at the time of diagnosis, at the time of first remission in the remission group and at the initiation of IFX therapy, and then 2 and 6 wk later in the relapsed groups. From peripheral blood mononuclear cells (PBMCs), the identification of markers (6B11, CCR4, CD3, CD4, CD8, CD11c, CD14, CD25, CD45RA, CD45RO, CD123; CD161, CXCR3, HLA-DR and Lin-1 BD Biosciences Pharmingen, San Diego, CA, USA; TLR-2 and TLR-4 eBioscience, San Diego, CA, USA) and FoxP3 assay (eBioscience, San Diego, CA, USA) were performed with a BD FACS Aria (BD Biosciences Pharmingen, San Diego, CA, USA)[51]. Briefly, from whole blood, PBMCs were separated with gradient centrifugation using Ficoll-Paque (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The isolated PBMCs were washed twice with Phosphate Buffered Saline pH 7.4 (PBS, Central Pharmacy of Semmelweis University, Budapest, Hungary) and were stained with the appropriate fluorescent antibodies according to the manufacturers' instructions. For intracellular staining, cells were incubated first with Fixation/Permeabilization Buffer (eBioscience, San Diego, CA, USA), then washed twice with Permeabilization Buffer (eBioscience, San Diego, CA, USA) and stained by FoxP3 and isotype control. At the end of staining, cells were washed twice with PBS and with Permeabilization Buffer for the detection of cell surface markers and FoxP3, respectively. Samples were resuspended in PBS and were measured within 1 h recording at least 50 000 events in the lymphocyte gate.

Our data did not follow normal distribution, therefore non-parametric statistical tests and median and interquartile ranges were used. Mann-Whitney and Friedman tests with Dunn's post hoc comparison and Spearman's correlation were used for statistical analysis, the level of significance was 5% ($P < 0.05$). All data are expressed as median (interquartile range). Statistical analysis was performed with Statistica 8 (Statsoft, Tulsa, OK, USA).

RESULTS

Major clinical characteristics and laboratory data are summarized in Table 1. The investigated cell prevalence values and cell ratios of the adaptive and innate immune systems are summarized in Tables 2 and 3, respectively. We also highlighted the most important alterations in Figures 1 and 2.

First, we compared the immune phenotype in therapy-naïve CD patients with that of healthy controls. In CD children, the prevalence of activated T cells (i.e. $CD4^+CD25^+$ cells) increased. At the same time, the prevalence of T cells with Th1 commitment (i.e. $CD4^+CXCR3^+$ cells) decreased resulting in a skewness of Th1/Th2 to Th2. The prevalence of memory (i.e. $CD4^+CD45RO^+$) cells increased and, therefore, a shift in the naïve/memory ratio toward memory cells was observed. The prevalence of regulatory T (i.e. $CD4^+CD25^{hi}FoxP3^+$) cells was comparable between the two groups.

Striking differences in cell prevalence values of innate immunity were obtained between therapy-naïve CD and healthy children. The occurrence of NK and NKT cells (marked as $CD3^-CD161^+$ and $CD3^+6b11^+$, respectively) was lower in CD children. Interestingly, the prevalence of the APCs investigated differed largely between CD and healthy children. DCs (i.e. those with $Lin1^-HLADR^+$ expression) were more prevalent and, within DC cells, the myeloid DCs (mDCs, i.e. $CD11c^+$ cells) were more prevalent, while plasmocytoid DCs (pDCs, i.e. $CD123^+$ cells) were less frequent in CD than in healthy children. This leads to skewness of mDCs in the mDC/pDC ratio. The prevalence of peripheral monocytes (i.e. $CD14^+$ cells) also increased in therapy-naïve CD patients. In addition, the prevalence of DC cells and monocytes expressing TLR-2 and TLR-4 receptors was also increased in CD. Of note,

Table 2 Prevalence and ratios of cellular members of adaptive immunity

Cell prevalence in parent population	Control	Therapy-naïve (before conventional therapy)	First remission (with conventional therapy)	Relapse (before IFX therapy)	IFX therapy (before 2nd infusion)	IFX therapy (before 3rd infusion)
$CD4^+$ in PBMC	35.16 (24.81-47.79)	36.52 (18.61-44.06)	37.42 (22.27-44.26)	39.12 (24.15-51.18)	39.77 (27.71-50.30)	35.65 (24.31-48.81)
Activated in $CD4^+$	5.92 (2.39-6.51)	10.61 (3.93-19.04)[a]	5.03 (3.37-7.93)[g]	7.84 (5.30-11.67)[a]	7.24 (5.67-12.25)[a]	9.98 (5.45-16.19)[b]
Naïve in $CD4^+$	64.57 (59.86-73.03)	63.36 (41.19-74.86)	61.31 (39.09-73.05)	66.31 (60.44-71.79)	64.47 (53.90-77.87)	58.11 (53.51-64.96)
Effector in $CD4^+$	21.91 (13.94-33.96)	31.29 (22.80-55.78)[a]	33.97 (28.54-39.44)[a]	29.47 (26.54-36.19)[a]	35.23 (27.74-40.36)[a]	37.62 (35.74-45.24)[b,i]
Naïve/effector ratio	2.73 (1.60-4.97)	1.57 (1.02-2.54)[a]	1.43 (1.14-2.21)[a]	2.34 (1.66-2.68)	1.79 (1.47-2.36)	1.52 (1.19-1.74)[a,e]
Th1 in $CD4^+$	16.19 (9.91-25.26)	6.49 (4.50-7.67)[d]	12.89 (5.12-19.03)[c]	10.9 (4.03-14.38)[a]	12.73 (8.12-15.81)	18.87 (12.51-38.11)[e]
Th2 in $CD4^+$	4.59 (2.78-5.51)	4.7 (1.98-6.03)	5.06 (1.42-8.48)	3.97 (2.27-7.19)	4.61 (2.55-5.65)	4.46 (2.11-8.16)
Th1/Th2 ratio	3.98 (3.06-5.12)	1.60 (0.50-2.11)[d]	1.95 (0.43-3.62)[b]	2.68 (0.85-5.07)	2.68 (1.42-6.14)	3.25 (1.37-4.80)[e]
Treg in $CD4^+$	1.25 (1.10-2.37)	1.36 (1.09-2.48)	1.41 (1.07-3.50)	1.31 (1.16-2.63)	1.33 (1.07-2.99)	1.96 (1.47-3.77)[b,e]

[a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.01$ *vs* control; [c]$P < 0.05$, [g]$P < 0.01$ *vs* therapy-naïve; [e]$P < 0.05$, [i]$P < 0.01$ *vs* relapse. Data are expressed as median (interquartile range). IFX: Infliximab; PBMC: Peripheral blood mononuclear cell; Activated ($CD25^+$); Naïve ($CD45RA^+$); Effector ($CD45RO^+$); Th1: T helper 1 committed, $CXCR3^+$; Th2: T helper 2 committed, $CCR4^+$; Treg: Regulatory T lymphocytes, $CD25^{hi}FoxP3^+$.

Table 3 Prevalence and ratios of cellular members of innate immunity

Cell prevalence in parent population	Control	Therapy-naïve (before conventional therapy)	First remission (with conventional therapy)	Relapse (before IFX therapy)	IFX therapy (before 2nd infusion)	IFX therapy (before 3rd infusion)
NKT in PBMC	1.39 (0.81-2.42)	0.7 (0.11-1.33)[a]	0.74 (0.37-1.34)	0.72 (0.45-1.23)	0.73 (0.37-1.20)	0.83 (0.31-2.05)
NK in PBMC	3.17 (1.71-4.76)	1.73 (0.72-3.49)[a]	1.98 (1.65-2.97)	1.95 (0.72-4.81)	2.14 (0.66-4.78)	2.45 (1.26-4.85)
DC in PBMC	0.61 (0.10-2.15)	1.05 (0.54-3.32)[a]	1.16 (0.58-2.98)[a]	1.45 (1.09-3.46)[a]	0.86 (0.30-2.80)	0.82 (0.52-2.31)
mDC in DC	46.04 (37.14-52.30)	61.66 (45.37-72.74)[b]	45.19 (37.83-56.68)[c]	69.01 (40.11-74.79)[a,e]	60.01 (48.03-68.50)[a]	57.39 (37.19-64.14)[a,g]
pDC in DC	27.38 (20.65-33.94)	19.26 (15.08-24.58)[a]	21.61 (14.76-32.61)[a]	18.33 (16.59-26.42)[a]	19.03 (17.26-32.38)	27.39 (19.68-36.70)[g]
mDC/pDC ratio	1.85 (0.44-1.46)	3.05 (2.32-6.11)[d]	2.35 (1.80-9.10)[b,c]	3.5 (1.74-7.79)[d]	3.04 (2.03-4.65)[d]	2.20 (1.66-5.54)[b,g]
TLR-2 in DC	8.8 (4.14-17.35)	55.08 (37.27-57.94)[d]	17.32 (6.32-32.54)[c]	38.7 (19.31-54.59)[b,e]	25.89 (5.69-60.43)	16.70 (2.29-42.61)[g]
TLR-4 in DC	2.24 (0.90-2.78)	14.36 (6.26-19.20)[b]	2.73 (1.35-7.21)[f]	10.11 (2.29-19.55)[a,e]	5.31 (2.81-13.42)	3.32 (0.10-6.05)[g]
Monocyte in PBMC	2.01 (1.38-3.82)	8.57 (3.82-16.72)[b]	5.96 (2.49-21.22)[a,c]	7.3 (2.65-15.44)[a]	5.58 (2.78-17.52)[a]	5.31 (3.54-6.94)[a]
TLR-2 in monocyte	16.44 (10.31-19.69)	29.65 (17.83-39.77)[a]	20.92 (7.87-29.15)[c]	20.63 (7.05-29.01)	19.89 (13.13-29.32)	18.52 (10.29-28.96)
TLR-4 in monocyte	7.17 (0.45-14.73)	14.59 (3.01-35.23)[a]	4.43 (1.82-8.31)[c]	10.39 (2.10-26.54)	8.26 (2.71-17.56)	6.30 (2.48-18.94)

[a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$ *vs* control; [c]$P < 0.05$, [f]$P < 0.01$ *vs* therapy-naïve; [e]$P < 0.05$ *vs* first remission; [g]$P < 0.05$ *vs* relapse. Data are expressed as median (interquartile range). IFX: Infliximab; PBMC: Peripheral blood mononuclear cell; NK: Natural killer, $CD3^-CD161^+$; NKT: Natural killer T, $CD3^+6b11^+$; DC: Dendritic cell, Lin1-HLA-DR^+; mDC: Myeloid dendritic cell, $CD11c^+$; pDC: Plasmocytoid dendritic cell, $CD123^+$; Monocyte ($CD14^+$); TLR: Toll-like receptor.

4 of the 14 therapy-naïve CD children did not respond to conventional therapy. Their immune phenotype at the therapy-naïve phase did not differ from those children who responded to conventional therapy (data not shown).

In the second phase of our study, we prospectively tested the alteration in cell prevalence values during therapy. At the time of first remission with conventional therapy, the Th1/Th2 ratio shifted to Th1 and normalized along with activated T cell prevalence. Memory T cells remained elevated, while all the other cell types of adaptive immunity were comparable to that measured before therapy. For innate immune cells, NK and NK-T remained lower than normal and total DC prevalence remained higher than the control. However, mDC and pDC ratios, total monocyte prevalence and cells expressing TLR-2/TLR-4 receptor values (including monocytes and DCs) were normal.

In children who relapsed with conventional therapy, immune phenotype again became comparable to that in therapy-naïve CD. Therefore, we measured lower Th1, increased activated T, higher DC and higher macrophage prevalence as well as higher TLR-2 and TLR-4 expression in comparison to controls. In addition, the prevalence of mDCs, simultaneously with TLR-2 and TLR-4 expressing DCs was higher in relapsed than in remitted CD. During IFX therapy, immune cell prevalence was measured at two time points (i.e. 2 and 6 wk after the initiation of therapy). Th1, activated T and Treg prevalence increased significantly by week 6 of therapy. Total DC, mDC, pDC, total monocyte, along with TLR-2 and TLR-4 expressing DC and macrophage prevalence were normal at this time. Of note, although no significant alteration was observed at week 2, some tendencies were already present (Figures 1 and 2).

DISCUSSION

In our study we investigated the major components of adaptive and innate immunity in a simultaneous manner in CD children. While CD4 numbers in therapy-naïve CD children were normal as in early studies with adult patients[52,53], we noticed a shift to the Th2 direction in Th1/Th2 committed T lymphocytes. This finding is in line with other reports on blood[29,30,32] or biopsy specimens of therapy-naïve CD children[31]. Similar to adults, we also found a higher than normal prevalence of activated $CD4^+$ cells and effector memory cells and a decrease in effector cell/naïve CD4 cell ratios[12-17]. We tested the idea that this was

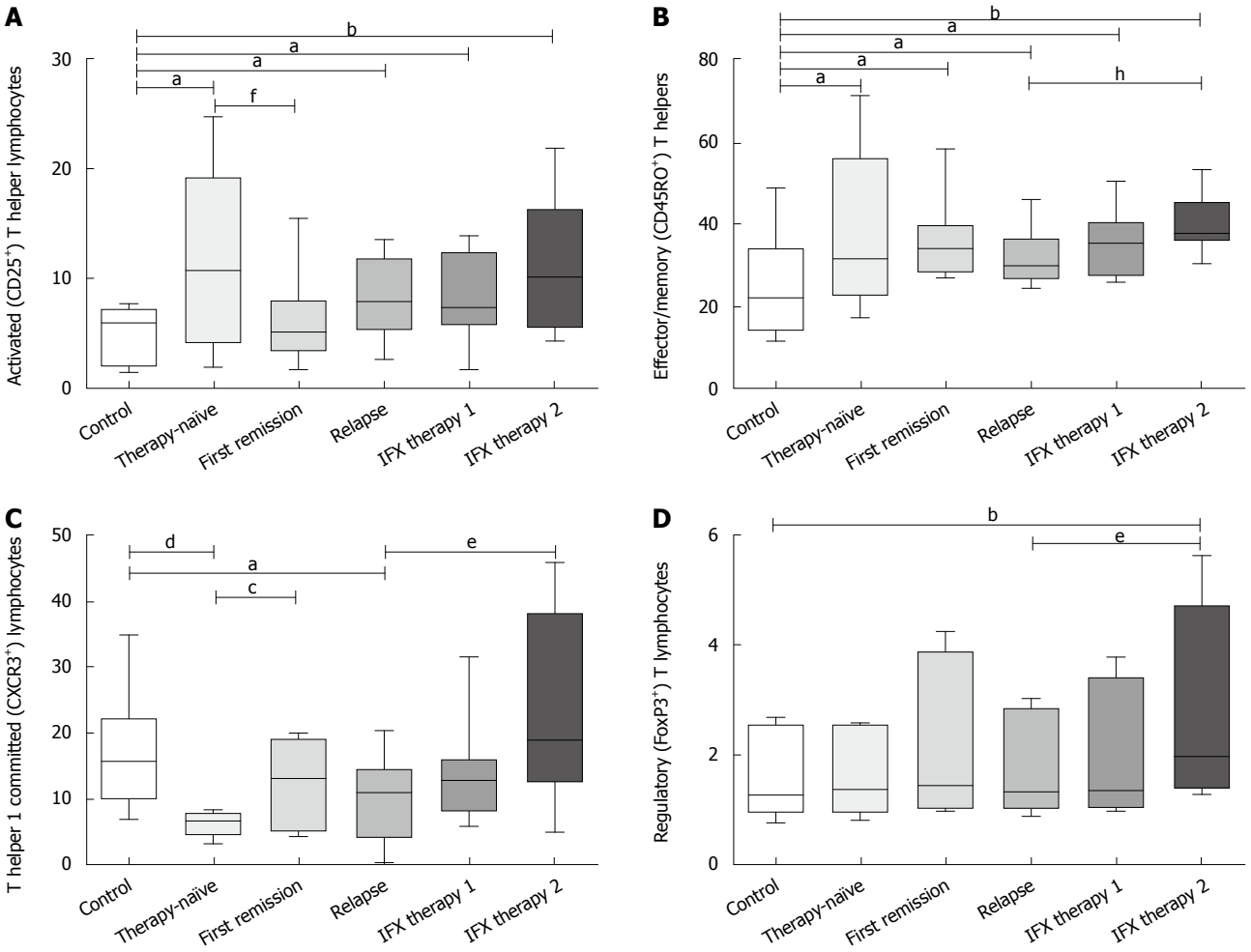

Figure 1 Prevalence of cellular members of adaptive immunity. The prevalence of activated (i.e. $CD4^+CD25^+$) (A), effector or memory (i.e. $CD4^+CXCR3^+$) (B), Th1 committed (i.e. $CD4^+CXCR3^+$) (C) and regulatory T (i.e. $CD4^+CD25hiFoxP3^+$) cells (D). $^aP < 0.05$, $^bP < 0.01$, $^dP < 0.001$ *vs* control; $^cP < 0.05$, $^fP < 0.01$ *vs* therapy-naïve; $^eP < 0.05$, $^hP < 0.01$ *vs* relapse.

due to low Treg numbers, but our data showing a normal prevalence of FoxP3 expressing CD4 cells do not support this notion. This finding is in contrast with that suggesting a diminution of Treg cells in untreated CD adults. However, in an earlier study Tregs were identified according to $CD4^+CD25^{high}$ positivity[8] currently not regarded as a sensitive marker for this cell type.

Our study provides novel information on the possible contribution of the innate immune system to a Th2 shift in therapy-naïve CD children. While some authors suggested that altered NK and NKT function may be a component in adult CD[17,26-28], our data are the first to show lower than normal NK and NKT prevalence in therapy-naïve CD children. In addition, we also observed a marked increase in monocyte and DC prevalence with an increase in the mDC/pDC ratio. These cell populations are major triggers of immune response and may be linked with the increase in memory T cell prevalence. As recent studies emphasized, mDC and pDC have distinct regulatory properties as mDC may shift the immune response not only toward Th1, but also in the Th2 direction[54], while pDC can induce Tregs[55]. Therefore, an increase in the mDC/pDC ratio in our patients may contribute to a lower Th1/Th2 ratio. Furthermore, we also measured an increased prevalence of TLR-2 and TLR-4 expressing monocytes and DCs that may also play a role in the activation of immune cells. This finding is in accordance with our previous observation of high TLR-2 and TLR-4 expression in the colonic mucosa of therapy-naïve CD children[34].

We tested prospectively the link between immune phenotype and disease activity index in our patients. The majority of immune system alterations in therapy-naïve CD are normalized with the normalization of PCDAI. While Th1 prevalence significantly increased compared to the therapy-naïve state and almost normalized during therapy, Th1/Th2 was still in the normal range at first remission suggesting a difference in CD immune phenotype between adults and children[4-7].

In remitted patients, NK and NKT prevalence increased and the difference between therapy-naïve CD and healthy controls disappeared. This finding does not support previous reports on decreased NK and NKT numbers in treated CD adults[17,26-28]. We also observed that the mDC/pDC ratio, prevalence of monocytes and that of TLR-2 and TLR-4 expressing DCs and monocytes were also normalized. This may suggest that normalization of immune phe-

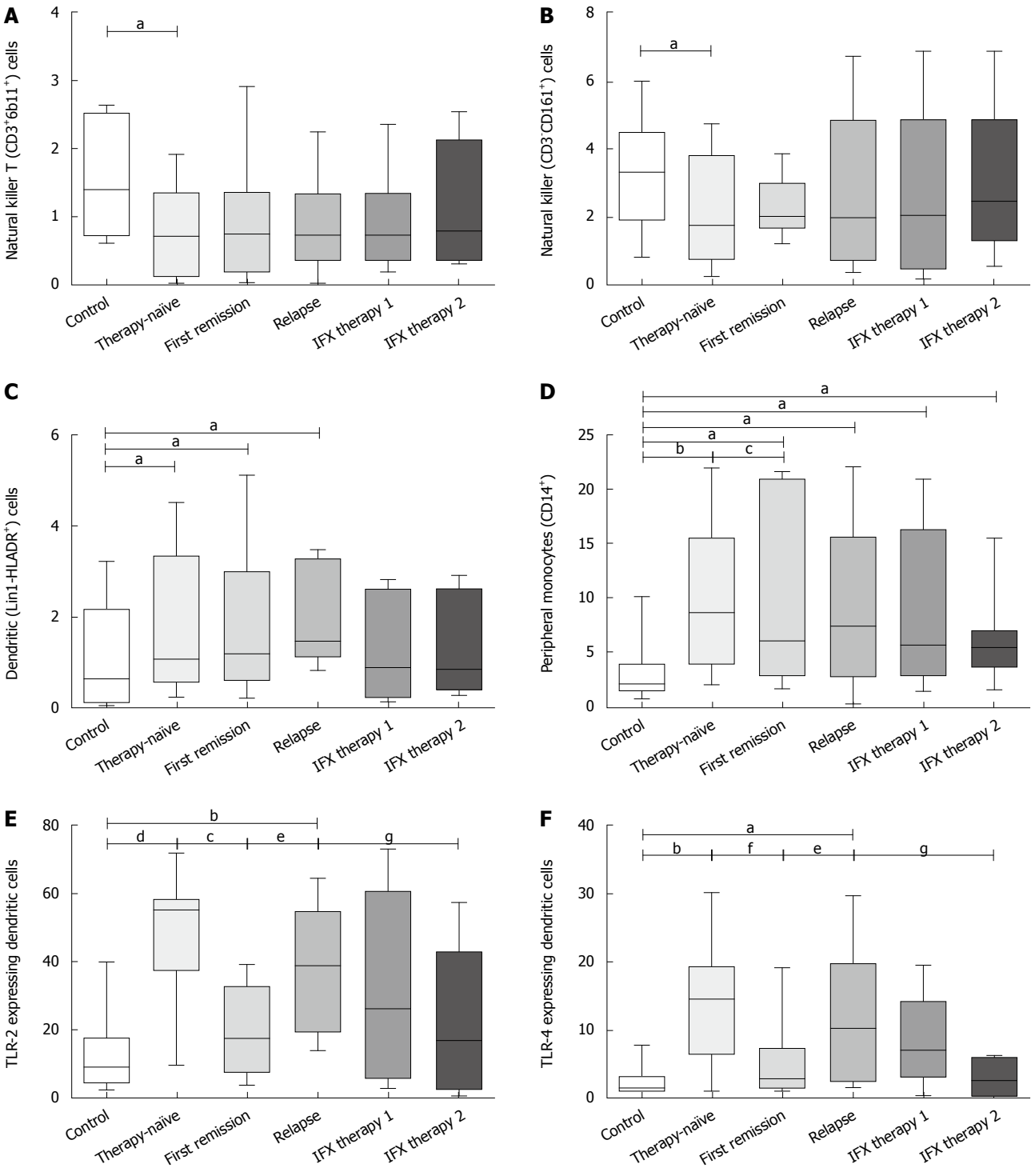

Figure 2 Prevalence of cellular members of innate immunity. The prevalence of natural killer T (i.e. $CD3^+6b11^+$) (A), natural killer (i.e. $CD3^-CD161^+$) (B), dendritic cell (i.e. $Lin1\text{-}HLA\text{-}DR^+$) (C), peripheral monocyte (i.e. $CD14^+$) (D), Toll-like receptor 2 expressing dendritic cell (E) and Toll-like receptor 4 expressing dendritic cell (F). $^aP < 0.05$, $^bP < 0.01$, $^dP < 0.001$ *vs* control; $^cP < 0.05$, $^fP < 0.01$ *vs* therapy-naïve; $^eP < 0.05$ *vs* first remission; $^gP < 0.05$ *vs* relapse.

notype is linked to an improvement in PCDAI. This may be a specific feature in childhood CD as a number of studies reported a higher than normal prevalence of activated and effector T lymphocytes even in treated CD adults[12-17]. Tregs probably do not play a role in this normalization as their prevalence was not altered in remission.

We performed another prospective study in 12 CD children who relapsed with conventional therapy and were treated with IFX. In these CD children, the immune phenotype was similar to that observed in therapy-naïve CD children (with the only exception of normal prevalence of NK and NKT cells). During IFX therapy, however, marked changes occurred in the 6th wk of IFX treatment and the prevalence of Th1 cells and the APCs investigated were normalized. Interestingly, the prevalence of activated T cells, memory cells and Treg cells were elevated further.

Recently, increased numbers of Th1 cells and high blood levels of Th1-type cytokines were found in adult patients with rheumatoid arthritis (RA) after IFX treatment[39,40]. Others also found an increase in effector and activated T cells with IFX[38,39]. While the explanation for this is still unclear, an attractive hypothesis may be that IFX inhibits the homing of Th1 and activated T cells to the inflammation site and transiently increases their peripheral occurrence[38,40].

Theoretically, this immune phenotype may refer to an increased risk for infections. However, our patients did not exhibit major clinical signs and symptoms of infection during IFX therapy. Interestingly, simultaneously with these changes, IFX therapy also increased the prevalence of peripheral Treg cells. This phenomenon - which may be due to a resistance of Tregs to IFX-induced apoptosis - was also demonstrated previously in the peripheral blood of adult CD patients[8], and in colonic samples from CD children[37].

In our patients, the prevalence of monocytes, mDCs, mDC/pDC ratio and TLR-2 and TLR-4 expressing DCs normalized during IFX therapy indicating the possible impact of biological therapy on innate immunity. This is in line with the observation of others investigating RA and CD patients[41,43-45].

Our study has three major limitations. First, although we did correct for multiplicity when performing pairwise comparisons, the study was not powered for the multitude of statistical tests we performed, thus some significances could occur by chance alone. Second, peripheral cell prevalence values do not necessarily reflect the intestinal phenotype. Third, our results may have been affected by ageing of the patients during the follow-up period. The short duration of our prospective study (i.e. about 10 mo until the first remission or 6 wk from the beginning of IFX therapy), however, makes this bias less likely.

CD exhibits several abnormalities in adaptive immunity (such as a decrease in Th1 cell and an increase in memory and activated T cell prevalence) and innate immunity (such as an increase in DC, monocyte and TLR-2 and TLR-4 exhibiting APC prevalence). The majority of the observed alterations of the innate immune system are normalized with the improvement of clinical signs and symptoms of CD, irrespective of whether this is obtained by conventional therapy or add-on IFX therapy. This finding suggests a link between immune phenotype and disease activity in childhood CD.

If these results are reinforced by other groups, the observations may raise the possibility that immune phenotype is a potential biomarker for clinical response in CD children.

COMMENTS

Background

About 10%-15% of patients with Crohn's disease (CD) are diagnosed before 18 years of age. Pediatric CD is a unique subtype of CD due to different location, altered responsiveness to therapy and different susceptibility factors compared with those in adult CD.

Research frontiers

Several studies indicate both the malfunction of adaptive and innate immunity in adulthood CD. Fewer data are available for CD children. The alteration of immune phenotype with treatment is also unclear in this population.

Innovations and breakthroughs

In this prospective study, the authors demonstrated marked alterations in both adaptive and innate immunity in childhood CD. These abnormalities were resolved in infants responding to conventional therapy but not in non-responding children. Immune phenotype depends on disease activity.

Applications

These observations support the involvement of members of the adaptive and innate immune systems in childhood CD. They also identify immune phenotype as a possible biomarker for the follow-up of therapeutic success.

Peer review

In this study, the authors prospectively investigated the major components of adaptive and innate immunity in CD children. The authors showed several abnormalities in adaptive and innate immunity in CD children. They also showed that the majority of observed alterations were normalized in remission stage and suggest a link between immune phenotype and disease activity in childhood CD. Although the number of CD patients enrolled to this study was small, this prospective study demonstrated clinically interesting results in CD children.

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BRIEF ARTICLE

Gastroesophageal flap valve status distinguishes clinical phenotypes of large hiatal hernia

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Abstract

AIM: To investigate two distinct clinical phenotypes of reflux esophagitis and intra-hernial ulcer (Cameron lesions) in patients with large hiatal hernias.

METHODS: A case series study was performed with 16831 patients who underwent diagnostic esophagogastroduodenoscopy for 2 years at an academic referral center. A hiatus diameter ⩾ 4 cm was defined as a large hernia. A sharp fold that surrounded the cardia was designated as an intact gastroesophageal flap valve (GEFV), and a loose fold or disappearance of the fold was classified as an impaired GEFV. We studied the associations between large hiatal hernias and the distinct clinical phenotypes (reflux esophagitis and Cameron lesions), and analyzed factors that distinguished the clinical phenotypes.

RESULTS: Large hiatal hernias were found in 49 (0.3%) of 16831 patients. Cameron lesions and reflux esophagitis were observed in 10% and 47% of these patients, and 0% and 8% of the patients without large hiatal hernias, which indicated significant associations between large hiatal hernias and these diseases. However, there was no coincidence of the two distinct disorders. Univariate analysis demonstrated significant associations between Cameron lesions and the clinico-endoscopic factors such as nonsteroidal anti-inflammatory drug (NSAID) intake (80% in Cameron lesion cases *vs* 18% in non-Cameron lesion cases, $P = 0.015$) and intact GEFV (100% in Cameron lesion cases *vs* 18% in non-Cameron lesion cases, $P = 0.0007$). In contrast, reflux esophagitis was linked with impaired GEFV (44% in reflux esophagitis cases *vs* 8% in non-reflux esophagitis cases, $P = 0.01$). Multivariate regression analysis confirmed these significant associations.

CONCLUSION: GEFV status and NSAID intake distinguish clinical phenotypes of large hiatal hernias. Cameron lesions are associated with intact GEFV and NSAID intake.

Key words: Large hiatal hernia; Reflux esophagitis; Cameron lesion; Gastroesophageal flap valve; Nonsteroidal anti-inflammatory drug

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INTRODUCTION

Hiatal hernia promotes gastric acid access to the esophagus and impairs its clearance. The overall consequence of increased esophageal acid exposure is reflux esophagitis. Larger hiatal hernias impair the normal anti-reflux mechanisms to a greater extent than do smaller hernias. Esophagitis severity and esophageal acid exposure increase significantly for hernias > 3 cm in length, as measured endoscopically[1]. The size of a hiatal hernia and the degree of lower sphincter hypotension are the most significant independent predictors of esophagitis presence and severity[2].

Gastric erosions or ulcers located on or near the neck of a large hiatal hernia sac, collectively referred to as Cameron lesions, cause gastrointestinal bleeding and anemia[3]. The prevalence of Cameron lesions is known to be dependent upon hiatal hernia size, with higher prevalence corresponding to larger hiatal hernia size[4].

There is convincing evidence that large hiatal hernias involve each of the two distinct disorders reflux esophagitis and Cameron lesions. However, the relationships between large hiatal hernias and these two disorders have not been clarified. We aimed to elucidate these relationships and to clarify the key factors that differentiate the clinical phenotypes of large hiatal hernias into reflux esophagitis and/or Cameron lesions.

MATERIALS AND METHODS

Patients and methods

From January 2005 to January 2007, 16 831 patients were referred for diagnostic esophagogastroduodenoscopy (EGD) to the Department of Endoscopy at Jikei University Hospital. Patients with the diagnosis of hiatal hernia were identified using databases of endoscopic and medical records. Endoscopic images of these patients were retrieved from the endoscopic filing system (Olympus Medical Systems, Tokyo, Japan), to confirm the presence of Cameron lesions and/or reflux esophagitis, as well as the presence and size of the hiatal hernia.

Cameron lesions were defined as gastric erosions or ulcers located on or near the neck of the hiatal hernia sac. Although Cameron lesions were originally described as linear erosions[3], various lesion shapes, namely, linear, oblong or round, have been reported[4]. Therefore, the shapes of the erosions or ulcers were not taken into account as long as the lesions were located on or near the neck of the hiatal hernia sac. Long linear erosions that extended from the neck to the lower or middle gastric body were excluded from this definition.

The standard endoscopic recording of diagnostic EGD comprised 40 still images, including two images of the gastric cardia from the retroflex views of the so-called U-turn and J-turn, and two images of the esophagogastric junction from the esophageal antegrade view. Additional still images were obtained with no limitation as to image numbers when abnormal findings, such as esophagitis and ulceration, were noted. Hiatal hernia size was defined as the diameter of the neck of the hernia sac in the still images of the retroflex views of the gastric cardia. For example, if the diameter of the neck was three times that of the endoscope shaft (the diameters of the endoscopes used ranged from 8.9 mm to 10.4 mm; GIF-Q260, GIF-XQ260, GIF-H260, GIF-Q240, GIF-XQ240; Olympus Medical Systems), the size of the hiatal hernia was recorded as 3 cm (Figure 1). The measurements were made at 0.5 cm intervals. A large hiatal hernia was tentatively defined as having a diameter ⩾ 4 cm.

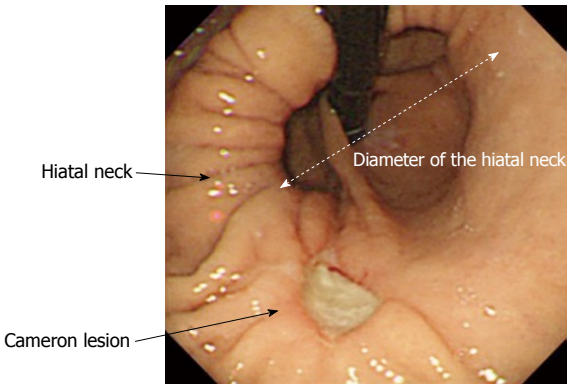

Figure 1 Representative image of the retroflex view of the gastric cardia in a patient with a large hiatal hernia and Cameron lesions.

A fold or ridge that surrounded the gastric cardia was designated as a gastroesophageal flap valve (GEFV). GEFV status was assessed using the still images of the retroflex views of the gastric cardia. A GEFV composed of a sharp fold that firmly surrounded the cardia was defined as an intact GEFV, which corresponded to grade I in Hill's classification[5,6]. A loose and dull fold or disappearance of the fold was classified as an impaired GEFV, which corresponded to grade Ⅱ-Ⅵ (Figure 2).

Reflux esophagitis was evaluated using the Los Angeles classification[7]. Patients with mucosal breaks were defined as having reflux esophagitis (grade A-D). The status of gastric mucosal atrophy was assessed using the Kimura-Takemoto classification[8]. The closed and open types were defined according to the presence of gastric mucosal atrophy.

GEFV status, hernia size, and the existence of reflux esophagitis and Cameron lesions were evaluated separately by three endoscopists, who were blinded to the clinical information. When there was a lack of consensus among the three endoscopists regarding the results obtained for the endoscopic factors, the matching results (if available) from two of the three endoscopists were adopted. If there were no matched results between the three endoscopists, they re-examined together the endoscopic still images and reached a consensus.

Statistical analysis

The parameters of age, sex, hiatus diameter, GEFV status, long-term nonsteroidal anti-inflammatory drug (NSAID) intake, gastric atrophy, and hemoglobin level were examined for associations with Cameron lesions and reflux esophagitis. Long-term NSAID intake was defined as the

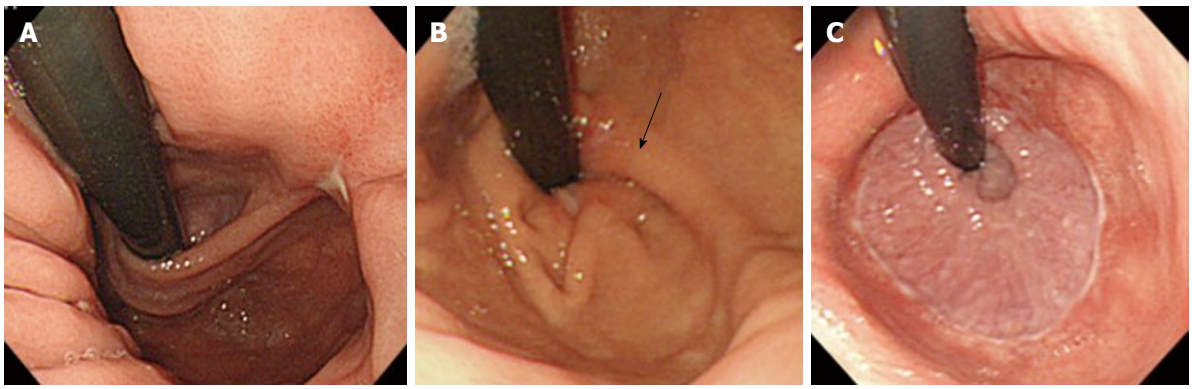

Figure 2 Representative endoscopic images of an intact gastroesophageal flap valve (A) and an impaired gastroesophageal flap valve (B, C). A loose and dull fold was classified as an impaired gastroesophageal flap valve (arrow in B).

use of any NSAID, including low-dose aspirin, for at least 3 d a week in the previous month. The associations were evaluated by univariate and multivariate logistic regression analyses. When the *P*-value was less than 0.05, the difference was considered to be statistically significant. All analyses were performed using the STATA 8.0 software (STATA Inc., College Station, TX, USA).

RESULTS

Associations between large hiatal hernias and the two clinical phenotypes

Of the 16 831 patients who underwent EGD, 4658 (27.7%) were identified as having hiatal hernias, and 49 (0.3%; 24 male and 25 female) met the criteria for large hiatal hernias. The mean age of the patients with large hiatal hernias was 69.8 years (range: 41-90 years). The indications for EGD for the 49 patients were as follows: epigastralgia (n = 12), heartburn (n = 11), anemia (n = 6), melena or hematemesis (n = 4), dysphagia (n = 2), vomiting (n = 2), and others (n = 12).

Cameron lesions and reflux esophagitis were found in five (10%) and 24 (49%) of the patients with large hiatal hernias, respectively. In contrast, Cameron lesions and reflux esophagitis were found in 0 (0%) and 1253 (8.4%) of the patients without large hiatal hernias, which indicated significant associations between large hiatal hernias and these two disorders. However, Cameron lesions concomitant with reflux esophagitis were not detected in any of the patients with large hiatal hernias.

Univariate and multivariate regression analyses of the associations between Cameron lesions and clinico-endoscopic factors

The results are shown in Table 1. Univariate analysis demonstrated significant associations between Cameron lesions and the clinico-endoscopic factors of NSAID intake, intact GEFV, and hemoglobin level in the 49 patients with large hiatal hernias. Although reflux esophagitis was found in 54.5% of the patients without Cameron lesions, it was not found in any of the patients with such lesions. Multivariate regression analysis showed a significant association between the presence of Cameron lesions and NSAID intake. As all of the patients with Cameron lesions showed intact GEFV, the association between Cameron lesions and GEFV status could not be evaluated adequately in the multivariate regression analysis.

Table 1 Univariate regression analysis and multivariate logistic regression analysis on the linkage between Cameron lesion and clinico-endoscopic factors

	Univariate regression		Multivariate logistic regression	
	Cameron	Non-Cameron	Odds ratio	95% CI
No. of patients	5	44	-	-
Age (mean ± SD, yr)	68-84 (77.2 ± 6.4)	41-90 (77.0 ± 11.9)	-	-
Gender (male:female)	0:5	24:20	-	-
Gastric atrophy (%)	0.4	0.5	-	-
Erosive esophagitis (%)	0	0.545	-	-
Intact GEFV (%)	100	18.1[b]	Can not be evaluated	-
NSAID intake (%)	80	18.2[a]	12.0[a]	1.07-134.1[a]
Hemoglobin level (g/dL)	7.2 ± 2.2[a]	12.7 ± 1.8[a]	-	-

[a]$P < 0.05$, [b]$P < 0.01$ *vs* Cameron lesion. GEFV: Gastroesophageal flap valve; NSAID: Nonsteroidal anti-inflammatory drug.

Univariate and multivariate regression analyses of the associations between reflux esophagitis and clinico-endoscopic factors

The results are shown in Table 2. Univariate analysis demonstrated significant associations between reflux esophagitis and the clinico-endoscopic factors of sex and intact GEFV. Reflux esophagitis was frequently observed in male patients and in patients without intact GEFV. Multivariate regression analysis showed a significant reverse association between reflux esophagitis and intact GEFV.

Endoscopic features of Cameron lesions

The results are shown in Figure 3. Five patients with large hiatal hernias presented with Cameron lesions. Two of

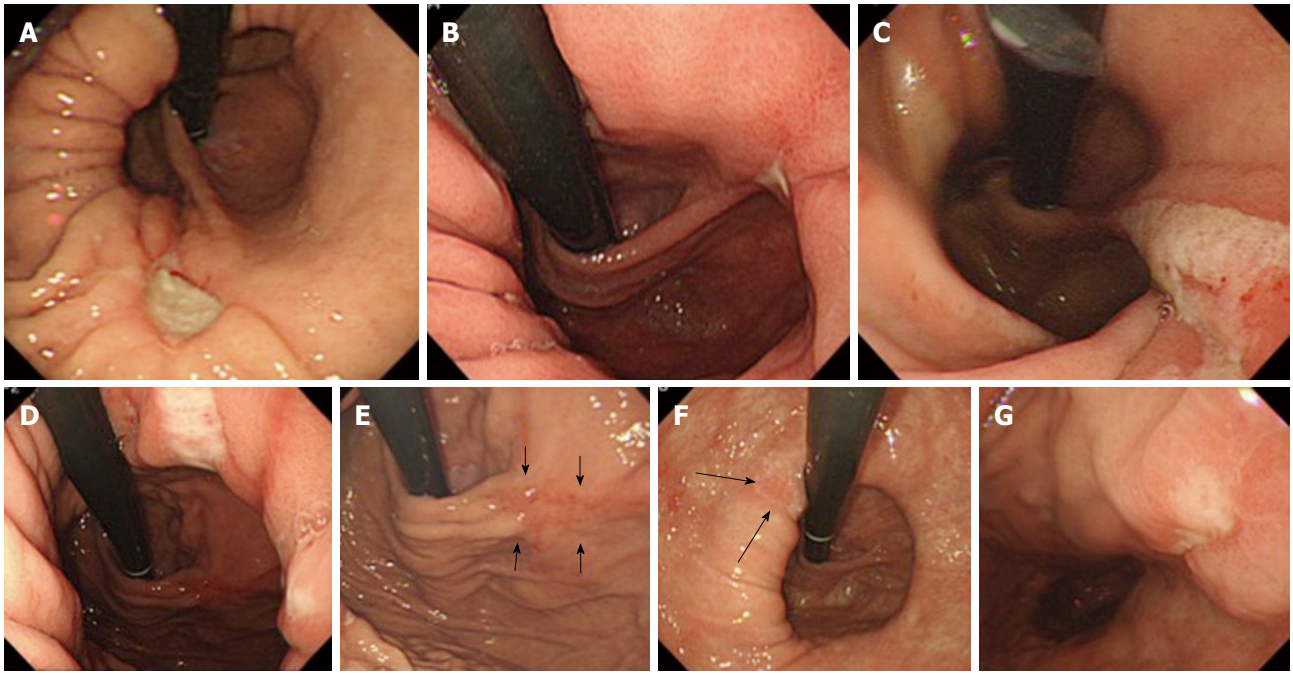

Figure 3 Representative endoscopic images of five patients with Cameron lesions. A: Case #1 (no nonsteroidal anti-inflammatory drug, Hb5.4); B: Case #2 (loxoprofen + low dose aspirin, Hb5.3); C: Case #3 (diclofenac, Hb6.3); D, E: Case #4 (low dose aspirin, Hb8.0); F, G: Case #5 (low dose aspirin, Hb10.3). An ulceration scar was noted at the point at which the gastroesophageal flap valve fold diverged from the anterior stomach wall (arrows in E and F).

Table 2 Univariate regression analysis and multivariate logistic regression analysis on the linkage between reflux esophagitis and clinico-endoscopic factors

	Univariate regression		Multivariate logistic regression	
	Reflux esophagitis	No esophagitis	Odds ratio	95% CI
No. of patients	24	25	-	-
Age (mean ± SD, yr)	41-87 (67.3 ± 14.1)	52-90 (72.2 ± 8.4)	-	-
Gender (male:female)	17:7	7:18[a]	2.66 (male)	0.73-9.7
Gastric atrophy (%)	34.6	64	-	-
Erosive esophagitis (%)	8.3	44[a]	0.17[a]	0.03-0.95[a]
Intact GEFV (%)	0	20	-	-
NSAID intake (%)	12.5	36	-	-
Hemoglobin level (g/dL)	12.5 ± 1.9	11.6 ± 2.9	-	-

[a]$P < 0.05$ *vs* reflux esophagitis. GEFV: Gastroesophageal flap valve; NSAID: Nonsteroidal anti-inflammatory drug.

these patients (#3 and #4) showed multiple linear erosions or ulcers that resembled the lesions described by Cameron *et al*[3]. In contrast, three of the patients showed solitary, non-linear lesions. In three patients (#1-#3) the lesions were localized to the crossing of the hiatal neck and the extension of the GEFV fold on the anterior gastric wall. In patient #4, there was no open ulcer at the crossing of the hiatal neck and the extension of the GEFV fold, although an ulceration scar was noted at the point at which the GEFV fold diverged from the anterior stomach wall (arrows in Figure 3).

DISCUSSION

Large hiatal hernias were identified in 0.3% of the patients referred to an academic referral center for diagnostic EGD. For this population, we found significant associations between the presence of large hiatal hernias and Cameron lesions or reflux esophagitis. These two disorders, which individually are linked with large hiatal hernias, were not detected concomitantly in any patient. GEFV status clearly differentiated the clinical phenotypes of large hiatal hernias into Cameron lesions or reflux esophagitis. Although the Cameron lesions were found exclusively in patients with intact GEFV, reflux esophagitis was frequently found in those with impaired GEFV.

GEFV contributes to the barrier functions against gastroesophageal reflux. The sling fibers of the stomach, which are located below the lower esophageal sphincter, are associated with a valve mechanism through which the pressure in the gastric fundus creates a flap that presses against the lower end of the esophagus[9]. GEFV status has been proposed as a useful predictor of gastroesophageal reflux disease[5,10,11]. In the present study, we have demonstrated that the anti-reflux mechanism of GEFV functions effectively, even in patients with large hiatal hernias, who are generally considered to have impaired barrier functions against gastroesophageal reflux.

All of the patients with Cameron lesions had intact GEFV, and 80% of the lesions were located at the point where the intact and sharp fold of the GEFV diverged from the stomach wall, especially on the anterior wall. As shown in Figure 1, the fold of the GEFV is speculated to be stretched excessively at the points where the fold

diverges from the stomach wall. Although the mechanisms that underlie the onset of Cameron lesions remain unclear, mucosal ischemia might result from the accumulation of excessive loads at a point on the hiatus neck and/or the extension of the GEFV. Mucosal injury might be exacerbated by the combination of mechanical loading and NSAID intake, which has been significantly linked with Cameron lesions.

Although *Helicobacter pylori* (*H. pylori*) is one of the main causes of gastric ulcer, the status was not examined systemically in this case series. We tested *H. pylori* status in four of five patients with Cameron lesions. Three and one of these four patients were negative and positive for *H. pylori*, respectively. Since the *H. pylori*-positive rate of the tested patients with Cameron lesions is lower than the rate (60%-80%) in elderly Japanese patients, *H. pylori* infection might not be the major ulcerogenic factor in Cameron lesions. One of the limitations in the present study is that we could not obtain the information on anti-secretory drug intake in all patients. Therefore, it is difficult to evaluate the association between anti-secretory drug intake and the clinical phenotypes seen in large hiatal hernia.

Cameron *et al*[3] have described multiple linear erosions on the gastric folds of the hiatus neck as a distinct entity. However, lesions of various shapes (i.e. linear, oblong or round) have been reported[4]. For three of the cases in the present study, the lesions were solitary and non-linear, and appeared to be different from the lesions demonstrated in the original article. Gastric erosions or ulcers located on the hiatus neck might have different etiologies. For example, multiple linear erosions or red streaks are often observed in other disorders, such as alkaline reflux gastritis, or in the antrum of the *H. pylori*-negative stomach[12]. Further studies with larger populations of patients with Cameron lesions might increase our understanding of the various etiologies of this disorder.

Endoscopic diagnosis of a hiatal hernia is not easy, and accurate measurement of hernial size is difficult[11,13]. Most investigators define hernial size as the distance between the Z-line and diaphragmatic crus using the length markings on the endoscope (most of the markings are at 5-cm intervals). This application of endoscopy has been shown to be unreliable owing to the large inter-observer variability[14]. It is also difficult to ensure that the tip of the endoscope is located precisely at the Z-line or diaphragmatic crus, as the circumferential distance from these landmarks to the incisors can vary. In cases of paraesophageal hernias without sliding hernias (type Ⅱ hiatus hernias)[15], measurements of this distance are not valid for assessing the size of an esophageal hernia. In the present study, which included type Ⅱ hiatus hernias, hernial size was defined as the diameter of the neck of the hernia sac in images taken of the retroflex views of the gastric cardia. The measurement of hiatus diameter by comparison with the endoscope shaft is relatively objective, and hiatus diameter might be valid for assessing the size of a type Ⅱ hiatus hernia. However, the hiatus diameter does not necessarily reflect the distance between the Z-line and diaphragmatic crus, even if these two parameters show strong correlation. Therefore, our definition of large hiatal hernias as having hiatus diameter $\geqslant$ 4 cm is tentative, and confirmation of the results obtained in the present study in prospective studies using both types of measurement is required.

In conclusion, GEFV status and NSAID intake differentiate the clinical phenotypes of patients with large hiatal hernias. Cameron lesions are found exclusively in patients with intact GEFV, and reflux esophagitis is frequently found in patients with impaired GEFV. NSAID intake and mechanical loading could contribute to the onset of Cameron lesions.

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COMMENTS

Background

Gastric erosions or ulcers, located on or near the neck of a large hiatal hernia sac, are designated as Cameron lesions. Large hiatal hernias are involved in reflux esophagitis and Cameron lesions.

Research frontiers

Cameron lesions and reflux esophagitis are mutually exclusive lesions that can be distinguished by the status of gastroesophageal flap valve (GEFV). Nonsteroidal anti-inflammatory drug (NSAID) intake is linked to the presence of Cameron lesions.

Innovations and breakthroughs

The authors have demonstrated that the anti-reflux mechanism of GEFV functions effectively even in a large hiatal hernia. Further investigations with manometer and pH monitoring might confirm the barrier function of GEFV and the etiology of Cameron lesions.

Applications

It is known that Cameron lesions recur frequently and cause chronic anemia. Conservative treatment such as anti-secretory drug and iron drug intake are effective for Cameron lesions. Sometimes blood transfusion might be necessary for treatment.

Peer review

This is an interesting paper about the association between hiatal hernia and pathological changes in esophageal and gastric mucosa represented by esophagitis and Cameron lesions. The authors also evaluated the effect of treatment with NSAID on the development of these mucosal changes. In addition, they have presented an alternative method for estimation of hiatal hernia size.

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BRIEF ARTICLE

Diagnosis and surgical treatment of primary hepatic lymphoma

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Abstract

AIM: To assess the benefits and limits of surgery for primary hepatic lymphoma (PHL), and probability of survival after postoperative chemotherapy.

METHODS: A retrospective analysis was undertaken to determine the results of surgical treatment of PHL over the past 8 years. Only nine patients underwent such treatment. The detailed data of diagnosis, treatment, and prognosis were carefully studied.

RESULTS: All patients were mistaken as having α-fetoprotein-negative hepatic cancer before pathological diagnosis. The mean delay time between initial symptoms and final diagnosis was 26.8 d (range: 14-47 d). Hepatitis B virus infection was noted in 33.3% of these patients. Most of the lesions were found to be restricted to a solitary hepatic mass. The surgical procedure performed was left hepatectomy in five cases, including left lateral segmentectomy in three. Right hepatectomy was performed in three cases and combined procedures in one. One patient died on the eighth day after surgery, secondary to hepatic insufficiency. The cumulative 6-mo, 1-year, and 2-year survival rates after hepatic surgery were, respectively, 85.7%, 71.4%, and 47.6%. One patient survived for > 5 years after surgery without any signs of recurrence until latest follow-up, who received routine postoperative chemotherapy every month for 2 years and then regular follow-up. By univariate analysis, postoperative chemotherapy was a significant prognostic factor that influenced survival (P = 0.006).

CONCLUSION: PHL is a rare entity that is often misdiagnosed, and has a potential association with chronic hepatitis B infection. The prognosis is variable, with good response to early surgery combined with postoperative chemotherapy in strictly selected patients.

Key words: Primary hepatic lymphoma; Diagnosis; Surgery; Survival

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INTRODUCTION

Primary hepatic lymphoma (PHL) is confined to the liver with no evidence of lymphomatous involvement in the spleen, lymph nodes, bone marrow, or other lymphoid structures[1]. PHL is a very rare malignancy, and constitutes about 0.016% of all cases of non-Hodgkin's lymphoma[1]. Most patients are treated with chemotherapy, with some physicians employing a multimodality approach[2]. However, the optimal therapy is still unclear and the outcomes

are uncertain. The purpose of this short report is to define the correct management of PHL with the help of nine clinical cases observed in the past 8 years.

MATERIALS AND METHODS

Patients

In view of the policy to try to resect hepatic lymphoma completely whenever possible, the charts of all patients operated upon for this condition from January 2002 to March 2010 were reviewed retrospectively. The diagnosis of PHL was defined[3]: (1) at the time of disease presentation, the patient's symptoms were caused mainly by the liver involvement; (2) there was an absence of palpable lymphadenopathy, and no radiological evidence of distant lymphadenopathy; and (3) there was an absence of leukemic blood involvement in the peripheral blood smear. Thus, patients with splenic, lymph node, or bone marrow involvement were excluded. During this period, only nine patients met these criteria. The analyses included sex, age, information concerning PHL, the type of liver surgery performed, postoperative therapy, the associated morbidity and mortality, and overall survival. The follow-up was complete for all nine patients.

Statistical analysis

The clinicopathological factors were analyzed for prognostic significance using a Kaplan-Meier product-limit method with a log-rank test. Significance was established at $P < 0.05$. Statistical calculations were performed by a statistical analysis program package (SPSS Inc., Chicago, IL, USA).

RESULTS

Clinicopathological data

The clinicopathological characteristics of the patients are depicted in Table 1. PHL commonly presents at 51 years of age (range: 28-78 years), with a male to female ratio of 0.8:1. The most common presenting symptom is abdominal pain or discomfort, which occurs in 55.6% of patients. Clinical examination revealed the presence of palpable abdominal masses in 22.2% of cases. All patients were misdiagnosed before pathological diagnosis. The mean delay time between initial symptoms and final pathological diagnosis was 26.8 d (range: 14-47 d). Diffuse large B-cell lymphomas were the most common type of PHL (66.7% of cases). The next most common type was mucosa-associated B-cell lymphoma, found in 22.2% of cases, followed by peripheral T-cell lymphoma (11.1%).

Table 1 Clinicopathological characteristics of patients, primary hepatic lymphoma and liver resection

Clinicopathological characteristics	Value (%)
No. of patients	9
Median age (at the time of surgery)	51.4 yr (range: 28-78 yr)
Sex	
Male	4
Female	5
Hypertension	1
Rheumatic heart disease	1
Hepatitis B infection	3 (33.3)
Hepatitis C infection	1 (11.1)
PHL	
Median diameter of PHL lesion	5.0 cm (range: 1.0-10.0 cm)
No. of PHL lesions	
Solitary	8
Multiple	1
Site of PHL lesion	
Left lobe	5
Right lobe	3
Bilobar	1
Vascular invasion	
Present	0
Absent	9
Lymph node involvement	
Present	1
Absent	8
Interruption of hepatic hilum	
Present	9 (14.0 ± 4.2 min, range: 8-20 min)
Absent	0
Type of liver resection	
Left hepatectomy	5
Right hepatectomy	3
Combined procedures	1
Length of operation	110 ± 59 (range: 50-255 min)
Blood loss of operation	211 ± 74 (range: 100-300 mL)
Adjuvant regional therapy	
Present	5 (55.6)
Absent	4 (44.4)

PHL: Primary hepatic lymphoma.

Laboratory findings

Liver function tests, including transaminases, alkaline phosphatase, lactate dehydrogenase and bilirubin were abnormal in 33.3% of patients. As the bone marrow was not involved, blood counts were within the normal range in most patients, except for three with marked splenomegaly related to liver dysfunction. Tests for human immunodeficiency virus, hepatitis B virus (HBV), and hepatitis C virus (HCV) were also carried out. In all patients, α-fetoprotein (AFP) and carcinoembryonic antigen were not significantly elevated. Hypercalcemia and coagulation abnormalities were not found in any of our patients. HBV infection was noted in 33.3% of these patients and hepatitis C in 11.1%.

Imaging studies

The most common presentation was a solitary lesion, which occurred in about 77.8% of cases, followed by multiple lesions in about 22.2% of patients. Ultrasound was performed in all the patients and usually demonstrated hypoechoic lesions to the surrounding normal liver parenchyma in 88.9% of cases.

On computed tomography, PHL lesions usually appear as hypoattenuating lesions, which might have a central area of low intensity that indicates necrosis (Figure 1A). Following the administration of intravenous contrast agent, PHL lesions might show slight enhancement (Figure 1B). Most magnetic resonance imaging (MRI) findings in PHL were described as hypointense on T1-weighted images, and hyperintense on T2-weighted images in our patients.

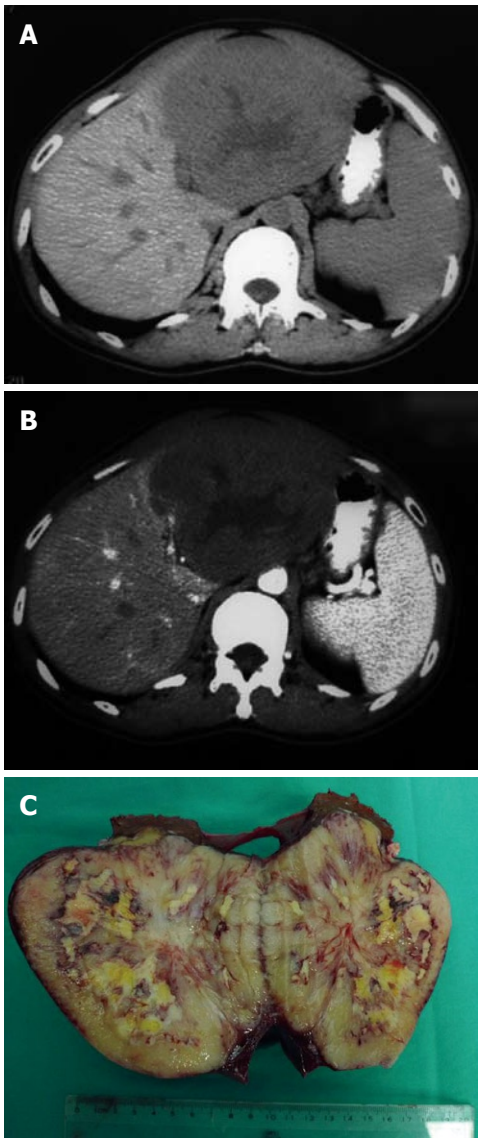

Figure 1 The preoperative imaging and surgical specimen in primary hepatic lymphoma. A: By computed tomography, primary hepatic lymphoma (PHL) appeared as a solitary hypoattenuating lesion with a central area of low intensity; B: PHL lesion was slightly enhanced following the administration of intravenous contrast agent; C: Pathological features revealed that the PHL lesion was an encapsulated, hypovascular tumor with central necrosis.

Operative details and perioperative outcome

Liver resection was performed in all cases. In eight patients, the resection was classified as curative, whereas one patient had palliative resection with a portal lymph-node-positive biopsy. The distribution of surgical procedures was left hepatectomy in five patients (55.6%), right hepatectomy in three (33.3%), and combined procedure in one (11.1%). In the postoperative course, one patient died on the eighth day after surgery, secondary to hepatic insufficiency. Postoperative chemotherapy was administered to five patients.

Survival and prognostic factors

The cumulative 6-mo, 1-year and 2-year survival rate was 77.8%, 66.7%, and 55.6%, respectively, with a median survival of 23 mo. One patient was also alive and tumor-free for > 5 years after resection. Among the factors considered, postoperative chemotherapy ($P = 0.006$) was the only significant prognostic factor for survival (Figure 2).

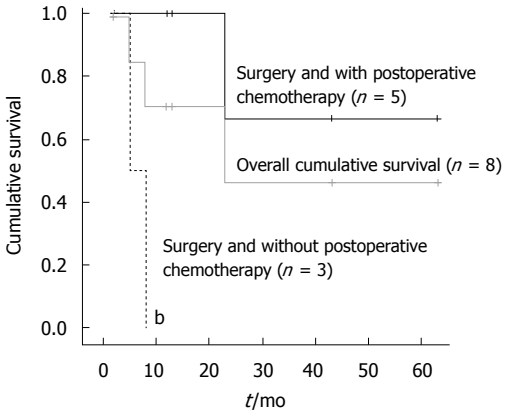

Figure 2 Survival among patients undergoing hepatic resection for primary hepatic lymphoma, with or without postoperative chemotherapy, and the overall cumulative survival of all patients (except for one who died perioperatively). $^{b}P < 0.01$ *vs* hepatic resection for primary hepatic lymphoma with postoperative chemotherapy (log rank test).

DISCUSSION

We suggest that our patients had PHL because we found only liver tumor and no lymphadenopathy or bone marrow lesions. Although the liver contains lymphoid tissue, host factors can make the liver a poor environment for the development of malignant lymphoma[4]. Therefore, PHL is rare. In our study, PHL in men was less frequent than in women, which did not accord with the previously reported trend towards male predominance[1-5].

The etiology of PHL is unknown, although several possible factors such as hepatitis, cirrhosis, and immunosuppressive drugs have been proposed[3]. There appeared to be a strong association between PHL and HBV in our patients. Hepatitis B was found in 33.3% of patients. In contrast, the prevalence of hepatitis C was very low, with only one patient having the infection. However, some authors have revealed a variety of possible associations between PHL and chronic HCV infection[6-8].

Conflicting theories exist on the association of HBV infection and PHL[3,9-11]. Aozasa *et al*[9] have reported a 20% prevalence of hepatitis B surface antigen positivity in a series of 69 patients with PHL, in which 52 patients were from western countries and 17 from Japan. Chronic antigenic stimulation by HBV has been postulated to play a role in the development of PHL[10]. Based on all these data, it is likely that some association between PHL and HBV does in fact exist. However, the high prevalence of HBV infection in the present study perhaps corresponded to the high HBV seropositivity in China of 10% in the general population[12].

Although it remains uncertain to what extent HBV contributes to the development of PHL, a host environment with impaired immunity might play an important role[6,7,13]. Therefore, we hypothesize that chronic HBV infection, as with our three patients, could have impaired

host immunity, which subsequently led to accelerated development of PHL. If this hypothesis is confirmed, suppression from HBV infection or from complications related to chronic viral infections could be crucial steps in preventing carcinogenesis of PHL.

Due to the rarity of this disease entity, the non-specific clinical presentation, and laboratory and radiological features, PHL can be confused with focal nodular hyperplasia, primary hepatic tumors, carcinoma with hepatic metastases, and systemic lymphoma with secondary hepatic involvement[10]. In the present study, all patients were mistaken as having AFP-negative hepatic cancer before pathological diagnosis. We retrospectively performed imaging studies to discriminate between these possibilities. As in our patients, most PHL is usually hypoechoic by sonography and shows hypointensity on T1-weighted and hyperintensity on T2-weighted MRI.

The optimal therapy for PHL is still unclear and the outcomes are uncertain[5]. Most patients with PHL present with poor prognostic features. One large review of 72 patients has shown the median survival to be 15.3 mo (range: 0-123.6 mo)[14]. In our study, the median overall survival for all nine patients was 23 mo. These results are better than those previously reported and could have been due to strict patient selection and the use of postoperative chemotherapy in most patients.

Our univariate analysis revealed that postoperative chemotherapy was the only significant prognostic factor that influenced survival. In a previous study, one patient treated with surgery followed by chemotherapy and radiation was reported to be alive at 5 years following initial diagnosis[2]. Page *et al*[15] have stratified several pretreatment risk factors after a retrospective cohort review. Lei has proposed that, in patients with localized disease, surgery followed by adjuvant chemotherapy should be considered to prevent disease recurrence[3]. Therefore, we advise that good prognosis of PHL can be obtained by early surgery combined with chemotherapy in strictly selected patients.

The primary limitation of this study was the small sample size and its retrospective nature, even though this study population represents a relatively large sample in surgical management of PHL in one single center. A larger prospective series could enable us to draw more substantive conclusions with regard to the roles of postoperative chemotherapy and HBV infection in the surgical setting of PHL.

COMMENTS

Background

Primary hepatic lymphoma (PHL) is a rare malignancy. The rarity of the disease causes problems in diagnosis and management. The optimal therapy is still unclear and the outcomes are uncertain.

Peer review

The authors reported their experience of surgical management of PHL. Satisfactory survival can be achieved by early surgery combined with postoperative chemotherapy in strictly selected patients.

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BRIEF ARTICLE

Meta-analysis of ADH1B and ALDH2 polymorphisms and esophageal cancer risk in China

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Abstract

AIM: To evaluate whether alcohol dehydrogenase-1B (ADH1B) His47Arg and aldehyde dehydrogenase-2 (ALDH2) Glu487Lys polymorphism is involved in the esophageal squamous cell carcinoma (ESCC) risk in Chinese Han population.

METHODS: Seven studies of ADH1B and ALDH2 genotypes in Chinese Han population in 1450 cases and 2459 controls were included for meta-analysis. Stratified analyses were carried out to determine the gene-alcohol and gene-gene interaction with ESCC risk. Potential sources of heterogeneity between studies were explored, and publication bias was also evaluated.

RESULTS: Individuals with ADH1B arginine (Arg)/Arg genotype showed 3.95-fold increased ESCC risk in the recessive genetic model [Arg/Arg *vs* Arg/histidine (His) + His/His: odds ratio (OR) = 3.95, 95% confidence interval (CI): 2.76-5.67]. Significant association was found in the dominant model for ALDH2 lysine (Lys) allele [glutamate (Glu)/Lys + Lys/Lys *vs* Glu/Glu: OR = 2.00, 95% CI: 1.54-2.61]. Compared with the non-alcoholics, Arg/Arg (OR = 25.20, 95% CI: 10.87-53.44) and Glu/Lys + Lys/Lys (OR = 21.47, 95% CI: 6.44-71.59) were found to interact with alcohol drinking to increase the ESCC risk. ADH1B Arg+ and ALDH2 Lys+ had a higher risk for ESCC (OR = 7.09, 95% CI: 2.16-23.33).

CONCLUSION: The genetic variations of ADH1B His47Arg and ALDH2 Glu487Lys are susceptible loci for ESCC in Chinese Han population and interact substantially with alcohol consumption. The individuals carrying both risky genotypes have a higher baseline risk of ESCC.

Key words: Esophageal cancer; Alcohol metabolizing enzyme genes; Polymorphism; Susceptibility

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INTRODUCTION

Esophageal carcinoma (EC) ranked as the eighth most common malignancy and the seventh leading cause of cancer death worldwide characterized by remarkable diversity according to geographical distribution. It is known that Asian countries, in particular China, Iran and Japan, have the highest incidence rates of EC in the world, and the esophageal squamous cell carcinoma (ESCC) is a predominant histological type. For example, in China, the age-standardized incidence rates of ESCC in males and females were 72-150/100000 and 26-64/100000, re-

spectively, from 1995 to 2004 in a southern population[1]. ESCC is a complex disease influenced by environmental as well as genetic factors[2]. Behavioral factors include alcohol drinking which is common to all "high-risk populations"[3]. Odds ratio for ESCC was 50.1 for those who were both heavy drinkers and smokers in comparison with those who neither drank nor smoked[4]. Alcohol is not a carcinogen, and acetaldehyde is the most toxic alcohol metabolite in alcohol-associated carcinogenesis[2].

Efficiency in the conversion of ethanol to acetaldehyde and subsequent oxidation to acetate depends mainly on the alcohol dehydrogenase-1B (ADH1B) and the aldehyde dehydrogenase-2 (ALDH2) activities. Upon consumption of an alcoholic beverage, ethanol is first catalytically oxidized into acetaldehyde, occurring mainly through ADH1B. It is subsequently metabolized into harmless acetate, chiefly by ALDH2. Thus, genetic variants that result in functional differences in enzyme activity, lead to differences in acetaldehyde exposure among drinkers. A polymorphism in the *ADH1B* gene, resulting in an amino acid transition from arginine (Arg) to histidine (His) at codon 47 (Arg47His) in exon 3, bestows the super-active "fast" metabolic character on ethanol. About a 40-times greater maximum velocity has been identified for the ADH1B fast His allele than that for the less active Arg/Arg form[5,6]. In contrast, ALDH2 has a polymorphism, which results from the substitution of glutamate (Glu) to lysine (Lys) at residue 487 (also recognized as Glu504Lys) encodes a catalytically inactive subunit of ALDH2, whose ALDH2 Glu/Lys genotype has only 6.25% of the normal ALDH2 Glu protein[7].

In China, the ADH1B 47His allele frequency decreases dramatically from 90% to 10% from East China to West China, and its geographic distribution is consistent with the unearthed culture relic sites of rice domestication in China[8,9]. The ALDH2 487Lys allele is essentially absent in all parts of the world except East Asia and has the highest frequency in China, and is high in south coast and east coast of China, and decreased gradually toward inland China, west, northwest and north China. The origin of ALDH2 487Lys could be traced back to ancient Pai-Yuei tribe in south China[10]. Combined with the demographic history, the ADH1B 47His and ALDH2 487Lys are carried by Han Chinese as they spread throughout East Asia[11]. One effect of the ALDH2 487Lys mutation is the "Asian flush" - the red face, nausea, and rapid heartbeat that many people with East Asian ancestry experience when they drink alcohol. The alcohol flushing response (Asian Glow) is a biomarker for ALDH2 487Lys allele[12].

Various studies have focused on ADH1B and ALDH2 polymorphisms and the risk of ESCC in Chinese Han population[13-19]. These studies also suggested a gene-gene and gene-alcohol interaction for ESCC risk[18]. For example, the risk for ADH1B Arg/Arg carriers was significantly increased from 1.2 to 74 times in non-drinkers and drinkers, respectively, compared with non-drinkers who carried the ADH1B His/His genotype[15]. A meta-analysis of seven Asian (Chinese, Japanese, and Thailand) studies found that 487Lys allele was risky for ESCC susceptibility[20]. However, there was only one Chinese population for the meta-analysis. Meta-analysis can provide an opportunity to help identify genetic associations by overcoming the coalescent issues[21]. We performed a meta-analysis among all eligible studies to clarify the effect of ADH1B Arg47His and ALDH2 Glu487Lys polymorphism alone and interactively on ESCC risk in Chinese Han population.

MATERIALS AND METHODS

Selection criteria and identification of eligible studies

Eligible studies were identified by searching the database of PubMed for relevant articles in English. The latest searches were undertaken in January 2010. The following search terms: "esophageal cancer" or "esophageal cancer" and "ADH2" or "aldehyde dehydrogenase" or "ADH1B" or "ALDH2" "aldehyde dehydrogenase 2" "polymorphism" were used in isolation and combination with one another. Searches were limited to the studies involving human subjects and Chinese population. All population-based case-control studies focusing on the associations between the ADH1B Arg47His, ALDH2 Glu487Lys variants and ESCC risk were eligible for inclusion. Review articles, case reports, esophageal adenocarcinoma and repeated literatures were excluded. When overlapping articles were found, we only included the publication that reported the most extensive information.

The full-text of the candidate articles were examined carefully to determine whether they accorded with the inclusion criteria for the meta-analysis. A total of seven published studies with full-text articles examined the association of polymorphisms ADH1B Arg48His and ALDH2 Glu487Lys, and 3 of the seven were about the interaction between *ADH1B* and *ALDH2* gene.

Data extraction

Data were extracted independently by Zhang GH and Mai RQ. We extracted the following information from each manuscript: author, year of publication, region, selection and characteristics of cancer cases and controls, and genotyping information.

Meta-analysis

The risk of ESCC associated with the ADH1B Arg47His and ALDH2 Glu487Lys variants were estimated for each study by odds ratio (OR) with 95% confidence intervals (CI) using comprehensive meta-analysis software version 2.0. For all studies, we evaluated the risk of the variant genotypes Arg/Arg or Arg/His, Lys/Lys or Glu/Lys and compared with the His/His and Glu/Glu genotypes. We then calculated the OR of the polymorphisms with dominant and recessive model. Between-study heterogeneity was estimated using the Cochran's Q and χ^2. Heterogeneity was considered statistically significant when $P < 0.05$. Point estimates and 95% CI were computed with both random effect and fixed effect models. If heterogeneity existed, point estimates and 95% CI were estimated on the basis of random effect model, Otherwise the fixed effect model was used. For the analysis of gene-gene interaction between ADH1B Arg47His and ALDH2 Glu487Lys, the His/His

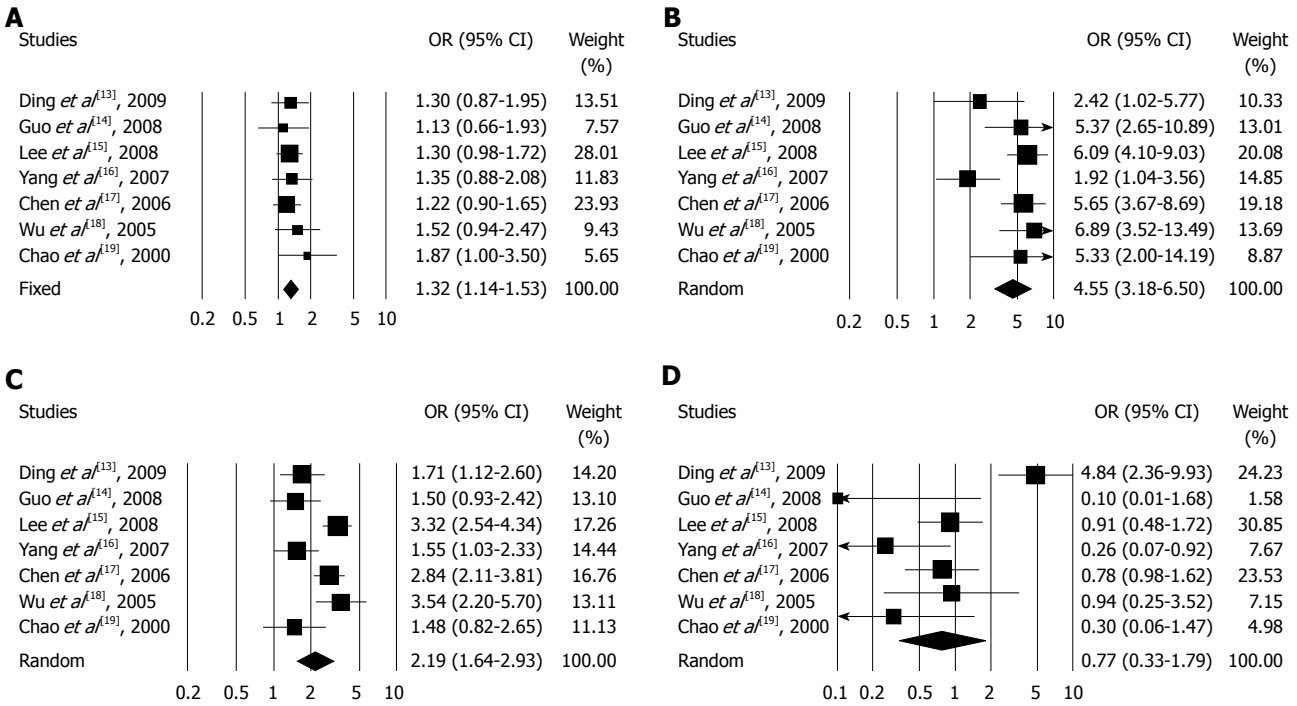

Figure 1 Forest plot shows the odds ratios and confidence intervals of the association. A: Between arginine (Arg)/histidine (His) and His/His genotype of alcohol dehydrogenase-1B (ADH1B) His47Arg polymorphism; B: Between Arg/Arg and His/His genotype of ADH1B gene His47Arg polymorphism; C: Between glutamate (Glu)/lysine (Lys) and Gly/Glu genotype of aldehyde dehydrogenase-2 (ALDH2) Glu487Lys polymorphism; D: Between Lys/Lys and Glu/Glu genotype of ALDH2 Glu487Lys polymorphism. OR: Odds ratio; CI: Confidence interval.

and Glu/Glu was considered as the reference genotype. For the gene-environment interaction, non-drinkers carrying the His/His genotype were set as compared subjects. Publication bias was assessed using funnel plots and Egger's test. $P < 0.05$ was considered statistically significant. χ^2 test was performed to examine the Hardy-Weinberg equilibrium (HWE) when genotype data were available.

RESULTS

Eligible studies and meta-analysis databases

Table 1 presents the characteristics of all the studies that were included in the meta-analyses. Seven studies published until July 2010 were about ADH1B Arg47His, ALDH2 Glu/Lys polymorphism and risk of ESCC, with a total number of 1450 cases and 2459 controls (Table 1). The genotype distribution in the control groups in each study did not depart from the HWE with $P > 0.05$.

Meta-analysis results

ADH1B Arg47His polymorphism: When the Arg/His and homozygote Arg/Arg were compared with the homozygous His/His genotype, the pooled ORs for all the 7 studies were 1.32 (95% CI: 1.14-1.53, $P < 0.001$, $P_{hetero} = 0.905$) in the fixed model and 4.55 (95% CI: 3.18-6.50, $P < 0.001$, $P_{hetero} = 0.029$) in the random model, respectively (Figure 1A and B). Similarly, increased associations were found in the dominant model, Arg/Arg + Arg/His *vs* His/His genotype, pooled OR was 1.62 (95% CI: 1.41-1.85, $P < 0.001$, $P_{hetero} = 0.206$). Arg/Arg *vs* Arg/His + His/His genotype, pooled OR was 3.95 (95% CI: 2.76-5.67, $P < 0.001$, $P_{hetero} = 0.014$) in the recessive model.

Table 1 Characteristics of studies included in meta-analysis

Authors	Origin	HWE of controls	*n*	Controls
Ding *et al*[13], 2009	Jiangsu	Yes	191	221
Guo *et al*[14], 2008	Gansu	Yes	80	480
Lee *et al*[15], 2008	Taiwan	Yes	406	656
Yang *et al*[16], 2007	Sichuan	Yes	191	198
Chen *et al*[17], 2006	Taiwan	Yes	330	592
Wu *et al*[18], 2005	Taiwan	Yes	134	237
Chao *et al*[19], 2000	Taiwan	Yes	88	105

HWE: Hardy-Weinberg equilibrium.

ALDH2 Glu487lys polymorphism: The variant heterozygous genotype Glu/Lys carriers have a significant increased risk of ESCC compared with those carrying Glu/Glu genotype (OR = 2.19, 95% CI: 1.64-2.93, $P < 0.001$, $P_{hetero} < 0.001$). But the homozygous genotype Lys/Lys did not show a significantly increased risk of ESCC (OR = 0.77, 95% CI: 0.33-1.79, $P = 0.537$, $P_{hetero} < 0.001$) (Figure 1C and D). Similarly, no associations were found in the recessive model (Lys/Lys *vs* Glu/Lys + Glu/Glu: OR = 0.48, 95% CI 0.19-1.20, $P = 0.118$, $P_{hetero} < 0.001$). Combining the homozygous Lys/Lys and heterozygous Glu/Lys genotypes, the pooled OR for the dominant model was 2.00 (Lys/Lys + Glu/Lys *vs* Glu/Glu: 95% CI: 1.54-2.61, $P < 0.001$, $P_{hetero} = 0.001$).

Gene-environmental interactions: The joint associations of the ADH1B, ALDH2 polymorphism and alcohol drinking with the risk of ESCC were observed when the analysis was stratified by alcohol drinking status. Among

Table 2 Interaction between alcohol drinking and genotype for esophageal squamous cell carcinoma risk

	Non-drinker			Drinker		
	Case/control	OR	95% CI	Case/control	OR	95% CI
ADH1B[13-15,17,18]						
His/His + Arg/His	217/1355	Ref.	-	674/668	6.21	2.39-16.27
Arg/Arg	22/72	2.37	1.40-4.01	258/61	25.20	10.87-53.44
ALDH2[13-18]						
Glu/Glu	96/604	Ref.	-	356/609	4.22	2.030-8.77
Glu/Lys + Lys/Lys	223/951	1.70	1.05-2.75	687/190	21.47	6.44-71.59

ADH1B: Alcohol dehydrogenase-1B; ALDH2: Aldehyde dehydrogenase-2; His: Histidine; Arg: Arginine; Glu: Glutamate; Lys: Lysine; OR: Odds ratio; CI: Confidence interval.

Table 3 Gene-gene interaction with alcohol dehydrogenase-1B and aldehyde dehydrogenase-2 for esophageal squamous cell carcinoma risk

Genotype[13,15,18]	Case	Control	*P* value	OR	95% CI
Glu/Glu and His/His	134	299	Ref	-	-
Glu/Glu and Arg+	301	196	0.0001	3.66	1.66-6.74
Lys+ and His/His	82	69	0.005	2.72	1.34-5.51
Lys+ and Arg+[1]	181	50	0.003	7.09	2.16-23.33

[1]Genotype data from[13,15,16,18]. His: Histidine; Arg: Arginine; Glu: Glutamate; Lys: Lysine; OR: Odds ratio; CI: Confidence interval.

non-drinkers, there was no evidence for an increased risk for ADH1B Arg/His *vs* His/His genotype of individuals (OR = 1.06, 95% CI: 0.75-1.50). However, compared with non-drinkers carrying ADH1B His/His genotype, drinkers carrying Arg/Arg and Arg/His genotype (OR = 6.97, 95% CI: 1.70-28.56) showed a significantly higher risk of ESCC, and the risk of homozygote Arg/Arg was highest (OR = 20.69, 95% CI: 5.09-84.13) among the subgroups of the three studies[13,15,17]. In the recessive model, when the drinking factor was integrated, statistically significantly elevated risks were found (Arg/Arg *vs* Arg/His + His/His: OR = 25.20, 95% CI: 10.87-53.44) (Table 2).

Odds ratios for the ALDH2 genotypes and the risk of ESCC were stratified by the alcohol consumption. Risk of ESCC was particularly high for drinkers with ALDH2 Glu/Lys genotype compared with non-drinkers having ALDH2 Glu/Glu genotype (OR = 23.83, 95% CI 3.75-151.05, $P < 0.001$) in the subgroups of five studies[13-15,17,18]. Furthermore, the homozygous Lys/Lys was found to interact with alcohol drinking to increase the ESCC risk (OR = 16.33, 95% CI: 5.21-51.17). In the dominant model, the OR risk was increased from 2.00 of null model to 21.47 of the interaction model.

Gene-gene interactions: Table 3 shows the interaction of ADH1B and ALDH2 genotypes on ESCC risk. Compared with subjects having ADH1B His/His with ALDH2 Glu/Glu, OR for those with ALDH2 Glu/Glu and ADH1B Arg+, ALDH2 Lys+ and ADH1B His/His, and ALDH2 Lys+ and ADH1B Arg+ was 3.66-13.46. The significantly increased risk for ESCC (OR = 7.09, 95% CI: 2.16-23.33, $P < 0.001$) was noted in individuals with ADH1B Arg+ and ALDH2 Lys+. Individuals carrying two risky alleles had an additional risk compared to those with only one risky allele.

Publication bias

Funnel plot and Egger's test were performed to assess the publication bias. No influence of publication bias was found in our study using Egger's test $P = 0.202$ for the ADH1B, Egger's test $P = 0.085$ for the ALDH2, as indicated by the funnel plot (Figure 2).

DISCUSSION

Several studies have demonstrated that the ADH1B and ALDH2 genotypes would be expected to result in exposure to high acetaldehyde concentrations[22]. A recent genome-wide association study showed that ADH1B Arg47His and ALDH2 Glu487Lys are associated with ESCC in a Japanese population[23]. The proportion of individuals carrying "susceptible genotypes of alcohol-related diseases" in Chinese Han healthy population was 68.16%[24]. In this meta-analysis, the pooled OR of ADH1B Arg47His polymorphism was 1.32 (95% CI: 1.14-1.53, $P < 0.001$, $P_{hetero} = 0.905$) and 4.55 (95% CI: 3.18-6.50, $P < 0.001$, $P_{hetero} = 0.029$) for Arg/His and homozygote Arg/Arg genotype in Chinese Han population, respectively. We also observed that ALDH2 Glu/Lys had independent and statistically significant effects on ESCC. Although there was no statistically significant increase of ESCC risk in the recessive model (OR = 0.48, 95% CI: 0.19-1.20, $P = 0.118$, $P_{hetero} < 0.001$) and Lys/Lys genotype (OR = 0.77, 95% CI: 0.33-1.79, $P = 0.537$, $P_{hetero} < 0.001$), the highest pooled OR was found in the Glu/Lys genotype (OR = 2.19, 95% CI: 1.64-2.93, $P < 0.001$, $P_{hetero} < 0.001$). Our meta-analysis confirmed a positive association between the polymorphisms of ADH1B, ALDH2 and susceptibility to ESCC. The study by Zhou *et al*[25] found that Glu/Lys genotype was a risk factor for esophageal squamous cell dysplasia. Combining with our results, ALDH2 may be a candidate biomarker to screen early ESCC.

ADH1B and ALDH2 polymorphism was considered as risk-conferring factor for alcohol dependence[26], and many studies have indicated that the synergistic interaction between ADH1B and ALDH2 polymorphism and alcohol drinking are involved in the risk of ESCC[14,17,18]. Taking alcohol consumption and genetic vulnerability into con-

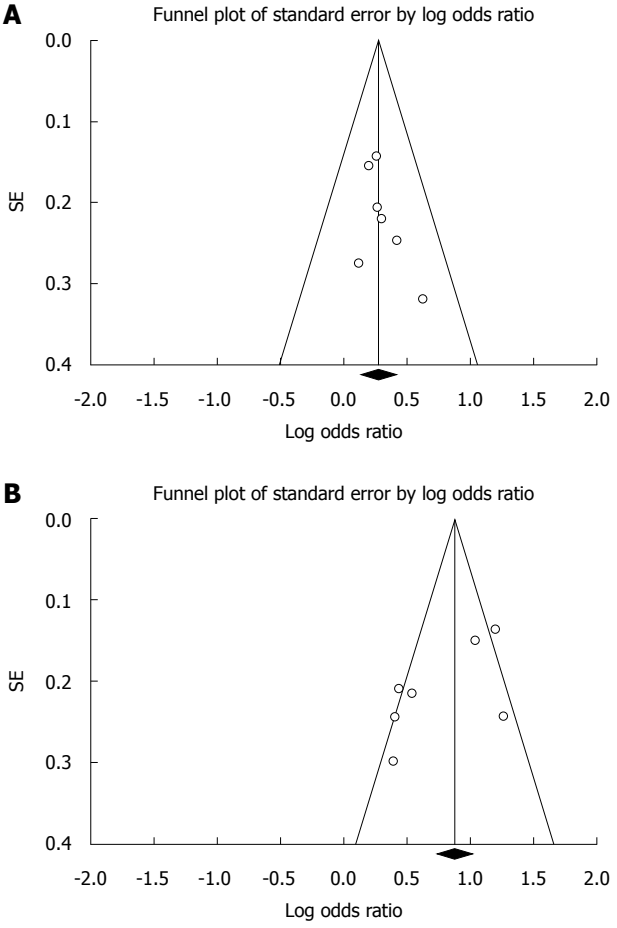

Figure 2 Funnel plot of alcohol dehydrogenase-1B His47Arg (A) and aldehyde dehydrogenase-2 Glu487Lys (B) polymorphisms and esophageal squamous cell carcinoma risk for publication bias.

sideration, this meta-analysis further identified an alcohol-genotype-dependent increase in ESCC risk for alcohol consumers. In this meta-analysis, the risk of individuals with ADH1B Arg/His genotype and alcohol drinking increased ESCC risk by 6.97 times (95% CI: 1.70-28.56). However, among non-drinkers, there is no strong evidence of an increased risk (OR = 1.06, 95% CI: 0.75-1.50). The magnitude of effect of ALDH2 Lys/Lys genotype was significant in alcohol drinkers (OR = 16.33, 95% CI: 5.21-51.17). Alcohol drinkers carrying ALDH2 Glu/Lys (OR = 23.83, 95% CI: 3.75-151.05) or ADH1B Arg/Arg (OR = 20.69, 95% CI: 5.09-84.13) had a higher risk of ESCC. These results might suggest that both the ADH1B Arg and ALDH2 Lys allele indicate a longer exposure to alcohol and highly-concentrated acetaldehyde, thus greatly increasing the susceptibility to ESCC. Alcohol has been intensified by the genetic modulation of ADH1B and ALDH2, which should make it more powerful. Furthermore, alcohol drinking with a combined genotype of ADH1B Arg/Arg and ALDH2 Glu/Lys was associated with increased DNA damage[27]. Avoidance of alcohol may be an important way to prevent ESCC among ADH1B Arg and ALDH2 Lys allele carriers.

There are many studies to explore the interaction between the ADH1B Arg/His and ALDH2 Glu487Lys polymorphism[14,15,18]. In this meta-analysis, we further found there was a highly significant gene-gene interactive effect between the two polymorphisms and ESCC risk. The risk effect of the interaction between ADH1B and ALDH2 was strongest in Arg+ and Lys+ carriers (OR: 7.09, 95% CI: 2.16-23.33), followed by Glu/Glu and Arg+ (OR = 3.66, 95% CI: 1.66-6.74) and Lys+ and His/His (OR = 2.72, 95% CI: 1.34-5.51) carriers. The results indicate that combined roles of the two genes and alcohol consumption should be considered to define the individual with ESCC risk.

We observed that the Arg/Arg and Glu/Lys genotypes were associated with increased risk for ESCC, but pooled OR was higher in carriers of the Arg/Arg (4.55) than in Glu/Lys carriers (2.00). Furthermore, the interactive risk of Glu/Glu and Arg+ (OR, 3.66) was higher than Lys+ and His/His (OR, 2.72). These results might suggest that the ADH1B has a predominant role in determining the risk of ESCC when the general population is considered.

Limitations

Although our primary result of this meta-analysis is suggestive, some limitations still exist. Firstly, we still lack more genotype data from the multiple highest incidence area, such as the Taihang Mountain in central China, Xinjiang and Chaoshan populations. Secondly, the number of studies included in this article was insufficient and the sample size of individual studies was also small. It is undoubted that larger studies should be done for confirming these findings, because our results are based on a limited sample size. Lastly, OR value was obtained without correction. More accurate OR should be corrected by age, gender and other environmental factors. A more precise analysis should be conducted if more detailed individual data are available, which would allow for an adjusted estimate. Adjusted covariates might help explain the association between ADH1B Arg47His and ALDH2 Glu/Lys polymorphism and susceptibility to ESCC.

In conclusion, this meta-analysis of seven case-control studies provided evidence that ADH1B Arg/His and ALDH2 Glu/Lys polymorphism was significantly associated with increased risk of ESCC. Gene-gene and gene-environment interactions are warranted to confirm the real contribution of these polymorphisms to ESCC susceptibility. Further studies in different high incidence areas of ESCC in China are also necessary to clarify the correlation between ADH1B Arg47His and ALDH2 Glu487Lys polymorphisms and ESCC risk.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is the fourth most frequent cause of cancer-related deaths in China. Alcohol intake is positively associated with the risk of ESCC. The activity of alcohol dehydrogenase 1B (ADH1B) and acetaldehyde dehydrogenase 2 (ALDH2) is primarily responsible for the oxidative detoxification, and polymorphism His47Arg in ADH1B and Glu487Lys in ALDH2 modulate the conversion rate of acetaldehyde. These polymorphisms are most prevalent in East Asia and they have highest frequencies in Chinese Han population.

Research frontiers

Many case-control studies have been performed to evaluate the ADH1B and ALDH2 polymorphisms for esophageal cancer risk in Chinese Han population,

including the role of gene-gene and gene-environment interaction in esophageal cancer risk. However, the evidence is insufficient with the small sample size.

Innovations and breakthroughs

This is the first meta-analysis which systemically studied the association between ADH1B and ALDH2 polymorphisms and esophageal cancer susceptibility in Chinese Han population, and suggested that ADH1B His47Arg and ALDH2 Glu487Lys are susceptible loci for ESCC and interact substantially with alcohol consumption. The individuals carrying the both genotypes have a higher baseline risk of ESCC.

Applications

This study provided a potential biomarker to identify high-risk individuals for esophageal cancer in Chinese Han population, specially among the alcohol drinkers.

Terminology

ADH1B: A member of the alcohol dehydrogenase family and the major enzyme that catalyzes alcohol to acetaldehyde in liver. ALDH2: A member of aldehyde dehydrogenases, and is the second enzyme of the major oxidative pathway of alcohol metabolism.

Peer review

The data and their interpretations are reasonable. This paper has a certain value in this research field because previous studies had a low statistical power because of the small sample size.

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BRIEF ARTICLE

Prognostic values of chromosome 18q microsatellite alterations in stage Ⅱ colonic carcinoma

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Abstract

AIM: To investigate the prognostic value of chromosome 18q microsatellite alterations (MA) in stage Ⅱ colon cancer.

METHODS: One hundred and six patients with sporadic stage Ⅱ colon cancer were enrolled in this study. DNA was extracted from formalin-fixed, paraffin-embedded tumor and adjacent normal mucosal tissue samples. MA, including loss of heterozygosity (LOH) and microsatellite instability (MSI), was analyzed by polymerase chain reaction, polyacrylamide gel-electrophoresis and DNA sequencing at 5 microsatellite loci on chromosome 18q (D18S474, D18S55, D18S58, D18S61 and D18S64).

RESULTS: Among the 102 patients eligible for MA information, the overall frequencies of LOH, high and low frequency MSI/microsatellite stable were 49.0%, 17.6% and 82.4%, respectively. The high frequency of 18q-LOH was significantly associated with the poor 5-year overall survival (OS) (P = 0.008) and disease free survival (P = 0.006). High levels of MSI were significantly associated with a longer 5-year OS (P = 0.045) while the higher frequency of 18q-LOH at the loci of D18S474 and D18S61 was significantly associated with a poorer 5-year OS (P = 0.010 and 0.005, respectively). But multivariate analysis showed that only the frequency of 18q-LOH was significantly associated with the prognosis of the disease.

CONCLUSION: High frequency of 18q-LOH is an independent prognostic factor indicating poor prognosis of the patients with stage Ⅱ colon cancer.

Key words: Chromosome 18q; Loss of heterozygosity; Microsatellite instability; Stage Ⅱ colon cancer; Prognosis

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INTRODUCTION

Colorectal cancer (CRC) is the third most common ma-

lignant cancer worldwide, with an estimate of one million new cases and a half million deaths annually[1]. Although the clinicopathological stage is currently the gold standard for prognosis, the molecular biological factors that determine the outcome of patients exhibiting the same clinicopathological stage are poorly understood, especially for stage Ⅱ and Ⅲ patients.

In recent years, multiple tumor-related biomarkers have been proposed as prognostic factors for CRC, but their predictive value has not been consistently demonstrated. Rapid advances in the molecular genetics of CRC have stimulated attempts to evaluate the prognostic significance of specific genetic alterations in this tumor type. The American Society of Clinical Oncology (ASCO) has published evidence-based clinical practice guidelines for the use of tumor markers in CRC bi-annually from 1996 to 2006[2]. Similarly, the European Group on Tumor Markers revised its recommendations for the use of tumor marker tests in the prevention, screening, treatment and surveillance of CRC in 2007[3]. These recommendations described new tumor markers, such as microsatellite instability (MSI), loss of heterozygosity (LOH) at chromosome 18q, as well as the traditional markers such as carcinoembryonic antigen (CEA), CA19-9, p53, Ras, and DNA ploidy.

It has been shown that CRC arises through at least two distinct pathways of genetic instability pathways of microsatellite alteration (MA): one involving chromosomal instability presenting as LOH, and the other involving MSI[4-6]. Although most studies have proven the prognostic value of LOH at chromosome 18q (18qLOH) in CRC, the outcomes are controversial. To further define the clinical effect of molecular genetic alterations on clinicopathological features and prognosis, we analyzed LOH and MSI at chromosome 18q in 106 patients retrospectively with stage Ⅱ colon cancer.

MATERIALS AND METHODS

This study consisted of randomly selected 106 consecutive patients who had undergone curative colon resection for sporadic stage Ⅱ colon cancer between 1996 and 2001. All cases were deemed sporadic, based on the absence of relevant family history in the initial patient interview records. None of the patients had additional synchronous colon cancers or other evidence of a heritable form of CRC.

A curative operation was defined as one in which no macroscopic tumor remained after surgery and histopathological examination showed radical resection margins were free of tumor at lines of the operative specimen. The methods used to exclude distant metastases at the time of resection included preoperative liver ultrasonography, abdominal and pelvic CT scan, chest X-ray, and intraoperative exploration. The right colon was defined as the large bowel proximal to the right half of the transverse colon; the left colon was defined as the large bowel between the right colon and the rectosigmoid junction. All specimens were histopathologically reviewed by a single gastrointestinal pathologist who was unaware of the results of molecular genetic testing. In accordance with the classification of tumors by the World Health Organization[7], we defined tumors as mucinous if 50% or more of the tumors displayed mucinous differentiation and as undifferentiated if features of tumor cell differentiation were absent. Other tumors were classified as adenocarcinomas.

Twelve lymph nodes on average were examined per case. Tumors were staged according to the TNM classification system and based on pathological findings after operation. Adjuvant chemotherapy was administered to patients with (1) clinicopathological factors with emergent presentation and associated with high risk of recurrence (i.e. bowel perforation or occlusion); (2) poorly differentiated tumors (histological grade); (3) deep tumor invasion and adjacent organ involvement (T4); (4) venous invasion; and (5) peritoneal involvement. The drug regimen for chemotherapy was 5-fluorouracil-based adjuvant chemotherapy for nearly 6 mo.

Patients were observed after the completion of therapy every 3 mo for 2 years, every 6 mo for the next 3 years, and then annually, or until relapse. History and physical examination, complete blood cell and platelet count, blood biochemistries, ultrasound, CEA and CA19-9 measurements were performed at each visit. The chest X-ray, colonoscopy, and CT scan were performed at least once a year.

The overall survival (OS) time was calculated from the date of surgery to the time of the last visit or death. The disease-free survival time was calculated from the date of resection to the time of relapse.

DNA extraction, LOH and MSI analysis

DNA extraction: Three to four 8 μm-thick sections were obtained from archival blocks of formalin-fixed, paraffin-embedded tumor and adjacent normal mucosal tissues. One 8 μm-thick section of each tumor sample was stained with HE, and the percentage of tumor cells was estimated by visual examination by a pathologist. Representative tumor samples contained a minimum of 80% of tumor cells. Microdissection of regions representing tumor and matching normal tissues was performed. Samples were selected and collected into Eppendorf tubes. The tissue samples were de-paraffinized with xylene, and the DNA was extracted by incubating the samples overnight at 56℃ in lysis buffer [10 mmol/L Tris-Cl (pH 8.0), 2.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.5% Tween 20] containing 0.2 mg/mL proteinase K (Invitrogen Inc., Carlsbad, CA). Digested products were boiled for 8 min at 95℃ to inactivate enzymes, centrifuged, and cooled on ice. The supernatant was used directly for polymerase chain reaction (PCR).

PCR: A panel of five polymorphic microsatellite markers located in chromosomal regions potentially involved in colon carcinoma development and progression were interrogated. These regions were D18S474 (18q21.1),

D18S55 (18q23), D18S58 (18q23), D18S61 (18q22.2), and D18S64 (18q21.3). The primer sets were obtained from published sequences and purchased from Invitrogen Inc. (Carlsbad, CA). The target sequences in normal and tumor DNA were amplified by PCR in a 50 μL reaction mixture containing 5 μL of DNA sample, 10 × buffer (10 mmol/L Tris-HCl pH 9.0, 50 mmol/L KCl, 0.1% Triton X-100), 3 mmol/L $MgCl_2$, 5 U *Taq* Polymerase, 10 mmol/L of each dNTP, and 10 mmol/L of each primer. Each forward primer was coupled with the fluorescent dye TET (4,7,2',7'-tetrachloro-6-carboxyfluorescein; Invitrogen Inc., Carlsbad, CA). The reactions were submitted to 35 cycles of amplification in a PTC-100™ thermal cycler (Bio-Rad Inc., America) at the following annealing temperatures: 55℃ for D18S474, 57℃ for D18S55, 61℃ for D18S58, 55℃ for D18S61, and 59℃ for D18S64. The presence and correct size of all amplicons were determined using 2% agarose gel electrophoresis (Biowest Inc., CA).

Fragment analysis: Amplicons were separated in a 6% polyacrylamide denaturing gel (Bio-Rad Inc., CA) using the ABI 377 DNA Sequencer (ABI Inc., CA). A total of 0.5 μL PCR product was mixed with 0.5 μL blue dextran loading solution (Bio-Rad Inc., CA). After 5 min of denaturation at 95℃, the sample was loaded onto the gel and subsequently run for two h at 2750 V, 125 W, and 50℃. After electrophoresis, the fluorescent signals were automatically collected by a gene scanner. Analysis of these signals with the Genotyper (Version 2.0) image analysis software (ABI Inc., CA) generated electropherograms, which displayed alleles as peaks. The height and area of the peaks calculated by the software were proportional to the concentration of the alleles in the sample.

LOH: The presence of two distinctly sized alleles in normal tissues was the necessary condition for evaluation of allelic losses. Cases in which the normal DNA sample was homozygous were classified as non-informative. The ratio of allele peak areas calculated for each tumor sample was divided by the allele peak area ratio of the normal matching control. If this quotient was greater than 1.00, the quotient was converted to give a result ranging from 0.00 to 1.00. Ratios below 0.6, indicating at least a 40% reduction of a tumor allele, were indicative of LOH[8,9].

MSI: The unique appearance of one or more alleles in the tumor DNA but not in its paired normal DNA, such as new peaks in the electropherogram, indicated the MSI[10]. To ensure the reproducibility of the results, DNA analysis of the first 20 samples was repeated and 99% concordance was recorded. Tumors were classified as demonstrating a high frequency of MSI (MSI-H) if instability was detected in more than two loci of the five interpretable microsatellite markers investigated. Tumors were deemed as low frequency MSI (MSI-L) if instability was found in one of the five markers according to the international criteria. Tumors without MSI were considered to be microsatellite stable (MSS)[11]. In our study, MSI-L and MSS tumors were considered as a single group in comparison with MSI-H tumors. However, further studies of the significance of MSI-L[12] are needed, which may represent a biologically and clinically distinct entity[13,14] (Figure 1).

Statistical analysis

Statistical analysis was performed using the SPSS 12.0 package. The main factors compared in this study were frequency of MA and survival. Contingency tables and the χ^2 test were used to evaluate differences between percentages. The association of disease-free and OS with prognostic factors was evaluated by means of multivariate logistic regression.

Disease-free intervals in patients with recurrence were measured as the interval between the date of resection and the date of diagnosis of recurrence. Duration of survival was measured from the date of resection until the date of death from any cause or until the censoring date of June 30, 2008. In the survival analysis, deaths caused by postoperative complications within 30 d were excluded. Survival curves were drawn according to the Kaplan-Meier method, and differences in disease-free and OS were evaluated by means of log-rank test. The simultaneous effects of more than one prognostic factors were estimated by the Cox's proportional hazards regression model. Mortality rate ratios were used to assess the difference in deaths caused by colon cancer. The significance level was set at 0.05; the confidence interval was 95%; and all *P* values reported were two-sided.

RESULTS

We investigated 66 male and 40 female patients. The mean age of this group was 57 years (range, 19-84 years). Of the 106 primary tumors, 47 were located in the right colon and 59 were located in the left colon. A total of 88 (83.0%) patients with the high recurrent clinicopathological factors mentioned above underwent adjuvant chemotherapy (Table 1).

Microsatellite alteration

The number of tumors that could be evaluated for microsatellites (MA) varied in region because the polymorphic markers were non-informative for some patients. 18qLOH was observed in 50 (49%) of the 102 patients. The incidence of 18qLOH was 30.2% (26 of 86), 23.4% (18 of 77), 28.6% (20 of 70), 35.0% (28 of 80), and 20.8% (15 of 72) at D18S474, D18S55, D18S58, D18S61 and D18S64, respectively. Of the 102 tumor specimens evaluated for MSI, 42 (41.2%) cases were identified as MSI in at least one locus. Furthermore, seven cases were MSI at three loci, 11 cases at two loci, and 24 cases at one locus. According to the criteria mentioned above, 18 cases (17.6%) that showed MSI in at least two loci were defined as MSI-H. The remaining 84 cases (82.4%) were defined as MSI-L/MSS (Table 2).

Poorly differentiated ($P = 0.023$) nonmucinous ($P = 0.005$) tumors located in the left colon ($P = 0.023$) more

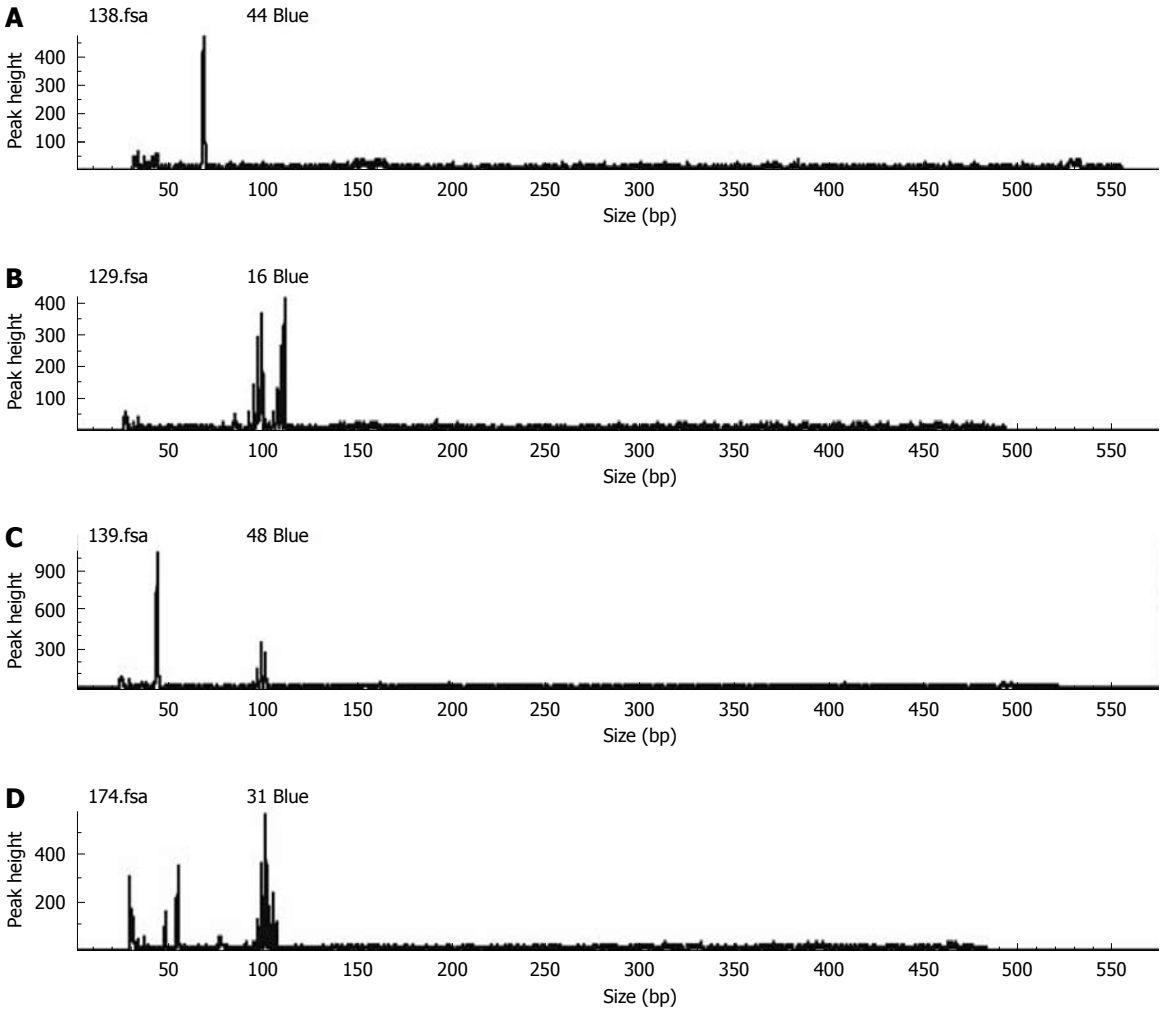

Figure 1 Representative examples showing homozygosity and heterozygosity in normal tissues and loss of heterozygosity and microsatellite instability in tumor tissues. A: Normal sample shows homozygosity; B: Normal sample shows heterozygosity; C: Tumor sample shows loss of heterozygosity; D: Tumor sample shows microsatellite instability.

likely displayed chromosome 18qLOH. No association was found between 18qLOH, age, and gender. MSI-H was more likely to be displayed by well/moderately differentiated ($P = 0.047$) mucinous ($P = 0.004$) tumors located in the right colon ($P < 0.001$). No association was found between MSI-H, age and gender (Table 3).

Survival analysis

Of the molecular markers tested, 18qLOH was significantly associated with a reduced 5-year OS ($P = 0.019$) and a disease-free survival ($P = 0.010$) during the follow-up period. High levels of MSI were moderately associated with an improved 5-year OS during the follow-up period ($P = 0.045$). However, the rates of disease-free survival were not significantly different between patients with MSI-H and MSI-L/MSS tumors (Table 4, Figure 2). When the status of chromosome 18q was evaluated according to each microsatellite locus, the D18S474 and D18S61 loci were associated with a poor 5-year OS ($P = 0.010$ and 0.005, respectively) (Table 5, Figure 3).

Cox's proportional hazards regression model was adjusted for gender, age, T stage and chemotherapy, and multiple markers were analyzed. Only 18qLOH was independently associated with the OS rate (RR = 2.679, $P = 0.021$). However, when 18qLOH was excluded from the proportional hazards regression model, MSI-H status was found to be associated with the OS (RR = 3.149, $P = 0.039$) (Tables 6 and 7).

DISCUSSION

In 2006, the ASCO published evidence-based clinical practice guidelines for the use of tumor markers in CRC to update the recommendations for the use of tumor marker tests in the prevention, screening, treatment, and surveillance of gastrointestinal cancers. As part of the update, 18q-LOH and MSI were mentioned in CRC for the first time[2]. In this publication, there were 16 series focusing on the LOH at 18q in the prognosis of early-stage CRC[15-30]. Of the 16 studies, 8 found that CRC patients with harbored LOH at 18q had a significantly lower survival than those who were heterozygous[15,19,20,22,25,28-30]. Four of the eight positive series also found that 18qLOH tumors had a significantly worse prognosis in a

Table 1 Clinical features of patients and survival status after surgery *n* (%)

Characteristics	Patients	Survival (mo) (mean ± SE)	3-yr overall survival (%)	5-yr overall survival (%)	*P* value
Gender					0.753
Male	66 (62.3)	104 ± 3	90.7	83.2	
Female	40 (37.7)	100 ± 3	91.6	84.1	
Age (yr)					0.011
≤ 60	60 (56.6)	107 ± 2	93.5	89.7	
> 60	46 (43.4)	97 ± 4	88.0	77.4	
Tumor site					0.467
Right colon	47 (44.3)	100 ± 3	89.5	82.9	
Left colon	59 (55.7)	104 ± 3	92.2	84.8	
Tumor size (cm)					0.213
< 5	48 (45.3)	108 ± 3	93.8	91.8	
≥ 5	58 (54.7)	101 ± 3	90.1	80.6	
Grade					0.220
Well/moderately differentiated	81 (76.4)	103 ± 2	92.0	85.3	
Poorly differentiated	25 (23.6)	104 ± 4	90.7	82.7	
Histologic features					0.246
Adenocarcinoma	79 (74.5)	104 ± 2	91.6	83.9	
Mucinous	27 (25.5)	101 ± 4	87.6	78.3	
pT stage					0.090
T3	63 (59.4)	106 ± 2	93.9	85.8	
T4	43 (40.6)	96 ± 4	87.0	78.5	
Chemotherapy					0.114
No	18 (17.0)	99 ± 3	83.3	69.8	
Yes	88 (83.0)	103 ± 4	90.9	84.8	

Table 2 Informative cases, loss of heterozygosity and microsatellite instability (*n* = 106) *n* (%)

Marker	Location	Informative cases	LOH (+)	MSI
D18S474	18q21.1	86	26 (30.2)	15 (17.4)
D18S55	18q23	77	18 (23.4)	11 (14.3)
D18S58	18q23	70	20 (28.6)	18 (25.7)
D18S61	18q22.2	80	28 (35.0)	13 (16.3)
D18S64	18q21.3	72	15 (20.8)	10 (13.9)
Overall	18q21-23	102	50 (49.0)	42 (41.2)

LOH: Loss of heterozygosity; MSI: Microsatellite instability.

multivariate analysis with hazard ratios for death of 2.0, 2.75 and 7.30[19,22,25], or for recurrence of 9.60[15]. Three reports did not find 18qLOH to be independently prognostic[20,28,30] and one did not perform multivariate analysis[29]. In two studies where 18qLOH was not found to be prognostic in a univariate or multivariate analysis[20,21], LOH at 18q did yield a poor prognosis in stage Ⅱ disease.

As for MSI, 17 series focusing on MSI in the prognosis of early stage CRC were considered in this review[15-17,19,31-43]. Six of the series were analyzed in patients on randomized therapeutic trials[16,17,19,31,40,41]. The remaining studies selected patients treated in defined time periods. Interestingly, 11 of the 17 series found that patients with MSI-H colon or rectal cancers had a significantly better survival than those with MSI-L/MSS[15,17,31,33-40,43]. Two series found no association of MSI-H with a better survival from randomized trials of adjuvant chemotherapy[16,19]. One study did find a statistically significant association of MSI-H status with a better disease-free survival[19]. Of the 11 positive series, six also found that MSI-H tumors had a significantly better prognosis in a multivariate analysis[17,33,37,38,40,43]. However, four did not find MSI-H to be independently prognostic[15,31,34,39]. Multivariate analysis was not done in one series[35].

Although there is evidence suggesting an association of 18qLOH and MSI with the prognosis of CRC, the ASCO expert panel determined that the data was insufficient and controversial to recommend using the two tumor markers as independent prognostic tests in the clinic.

Our study in patients with stage Ⅱ colon cancer confirms some previous findings of a lower survival in patients with 18qLOH and a longer survival in patients with MSI-H to some extent. Our present study showed that stage Ⅱ sporadic colon cancer patients with 18qLOH were associated with a significantly shorter OS and a disease-free survival as compared with 18qLOH-negative patients. This association was independent of other clinicopathological variables. According to other investigators, tumors with 18qLOH were more frequently located in the left colon. This observation, together with that of the worse prognosis of colon cancers with 18qLOH, may account for the findings that patients with tumors in the left side of the colon have a worse prognosis than those in the right side.

The reasons why some studies did not find that 18qLOH can predict survival in stage Ⅱ CRC may have several explanations. First, different loci and number of 18q markers were used by various investigators. Second, variation in surgical techniques, regimens of chemotherapy or radiotherapy, and methods of histopathologic analysis of the tumor (i.e. electrophoresis or DNA sequencing directly) could create significant differences. Third, differ-

Table 3 Correlation between loss of heterozygosity at chromosome 18q, microsatellite instability and clinicopathologic variables of patients *n* (%)

Characteristics	Cases	LOH (+)	LOH (-)	*P* value	MSI-H (%)	MSI-L/MSS (%)	*P* value
Gender				0.662			0.272
Male	63 (61.8)	27	36		9	54	
Female	39 (38.2)	23	16		9	30	
Age (yr)				0.435			0.111
≤ 60	58 (56.9)	32	26		11	47	
> 60	44 (43.1)	18	26		7	37	
Tumor site				0.023			< 0.001
Right colon	46 (45.1)	13	33		15	31	
Left colon	56 (54.9)	37	19		3	53	
Tumor size (cm)				0.232			0.327
< 5	48 (47.1)	28	20		8	40	
≥ 5	54 (52.9)	22	32		10	44	
Grade				0.016			0.047
Well/moderately differentiated	78 (76.5)	34	44		15	63	
Poorly differentiated	24 (23.5)	16	8		3	21	
Histologic features				0.005			0.004
Adenocarcinoma	77 (75.5)	44	33		5	72	
Mucinous	25 (24.5)	6	19		13	12	
pT stage				0.126			0.272
T3	61 (59.8)	33	28		11	50	
T4	41 (40.2)	17	24		7	34	
Chemotherapy				0.650			0.782
No	17 (16.7)	10	7		4	13	
Yes	85 (83.3)	40	45		14	71	

LOH: Loss of heterozygosity; MSI-L: Low frequency microsatellite instability; MSI-H: High frequency of microsatellite instability; MSS: Microsatellite stable.

Table 4 Five-year overall survival and disease-free survival after surgery in relation to microsatellite alteration in patients with stage Ⅱ colon cancer

Parameter	Cases	5-yr overall survival (%)	*P* value	5-yr disease-free survival (%)	*P* value
18q-LOH			0.019		0.010
(+)	50	71.3		70.5	
(-)	52	88.3		87.1	
MSI			0.045		0.155
MSI-H	18	94.4		88.5	
MSI-L/MSS	84	77.8		76.7	

18q-LOH: Loss of heterozygosity at chromosome 18q; MSI: Microsatellite instability; MSI-L: Low frequency MSI; MSI-H: High frequency of MSI; MSS: Microsatellite stable.

Table 5 Five-year overall survival after surgery in relation to each microsatellite locus

Marker	Cases	5-yr overall survival (%)	*P* value
D18S474	86		0.010
LOH (+)	26	65.2	
LOH (-)	60	84.9	
D18S55	77		0.504
LOH (+)	18	77.8	
LOH (-)	59	81.6	
D18S58	70		0.293
LOH (+)	20	71.4	
LOH (-)	50	83.3	
D18S61	80		0.005
LOH (+)	28	64.3	
LOH (-)	52	87.0	
D18S64	72		0.076
LOH (+)	15	69.0	
LOH (-)	57	86.3	

LOH: Loss of heterozygosity.

ences in TNM stage (stage Ⅱ or stage Ⅲ), T stage (T3 or T4), or tumor location (colon or rectum) could also contribute to the controversial results. For these reasons, our study selected a relatively small range of tumor locations (colon cancer) and TNM stage (stage Ⅱ) to investigate the frequency of 18qLOH and its prognostic value.

In contrast to the unfavorable prognosis associated with 18qLOH, MSI-H is associated with a favorable prognosis. Patients with tumors exhibiting MSI-H had a better OS than patients with MSS tumors. The correlation between the MSI-H genotype and disease-free survival was not significant ($P = 0.155$). However, we hypothesize that this association lacks significance only as a result of the small sample size examined. The favorable effect of MSI on survival, however, resulted as being independent only when multivariate analysis did not consider 18qLOH. The survival advantage of patients with MSI-H tumors compared to those with MSS tumors is not unanimously accepted. Thus, the outcome referred to this phenotype is controversial. In our study, the National Institute of Health consensus definition of MSI has been used.

The chromosome 18q contains several genes with potential importance in colon cancer pathogenesis and progression. Deletion of portions of 18q has been implicated

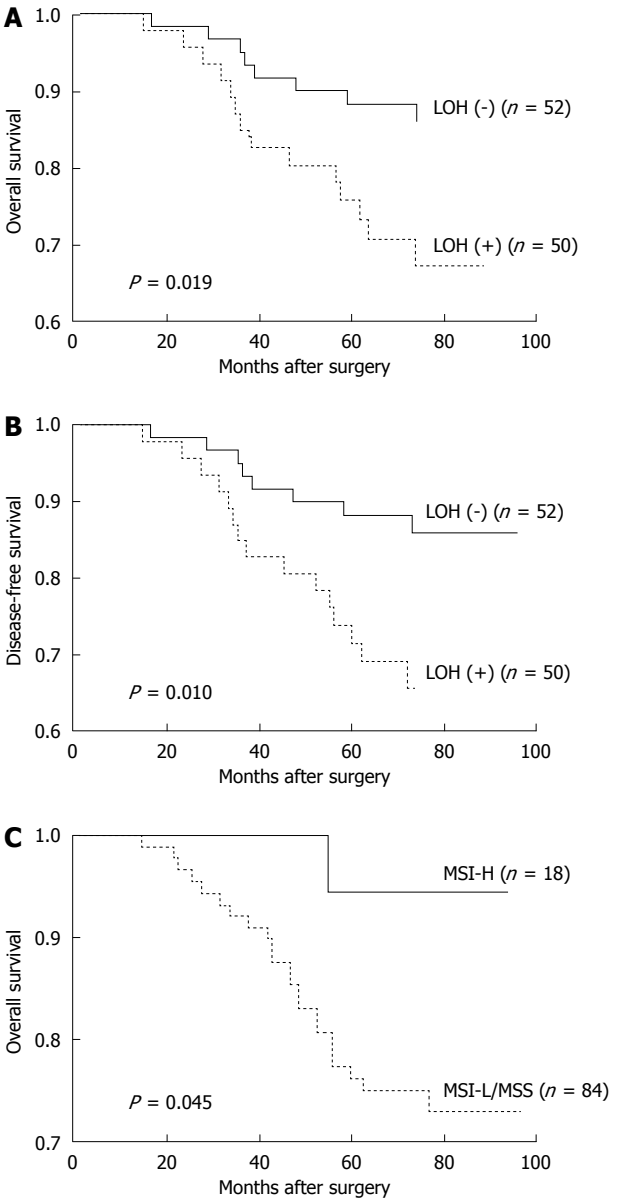

Figure 2 Kaplan-Meier estimates of overall survival and disease-free survival among patients with stage Ⅱ colon cancer according to microsatellite alteration. In our study, 18qLOH was significantly associated with reduced 5-year overall survival (P = 0.019) (A) and disease-free survival (P = 0.010) (B). High levels of microsatellite instability was moderately associated with improved 5-year overall survival during the follow-up period (P = 0.045) (C). LOH: Loss of heterozygosity; MSI-L: Low frequency microsatellite instability; MSI-H: High frequency of microsatellite instability; MSS: Microsatellite stable.

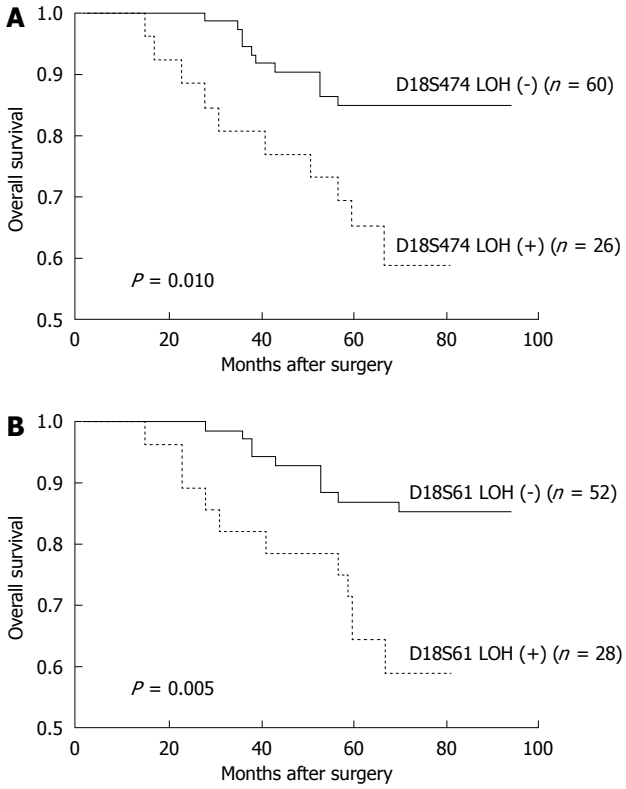

Figure 3 Kaplan-Meier estimates of overall survival among patients with stage Ⅱ colon cancer at microsatellite loci, D18S474 and D18S61. When the status of chromosome 18q was evaluated according to each microsatellite loci, the D18S474 (A) and D18S61 (B) loci were associated with worse 5-year overall survival (P = 0.010 and 0.005, respectively). LOH: Loss of heterozygosity.

Table 6 Univariate analysis of prognostic factors for 5-year survival according to Cox's proportional hazards regression model in patients with stage Ⅱ colon cancer

Variables	B	SE	Wald value	*P* value	RR
Age	-0.837	0.404	4.290	0.038	0.433
pT stage	0.667	0.395	2.859	0.091	1.949
Chemotherapy	-0.727	0.443	2.698	0.100	0.483
18q-LOH	0.903	0.425	4.509	0.014	2.467
MSI	1.147	0.736	2.428	0.039	3.149

18q-LOH: Loss of heterozygosity at chromosome 18q; MSI: Microsatellite instability.

Table 7 Multivariate analysis of prognostic factors for 5-year survival according to Cox's proportional hazards regression model in patients with stage Ⅱ colon cancer

Variables	B	SE	Wald value	*P* value	RR
18q-LOH	0.985	0.429	5.286	0.021	2.679

18q-LOH: Loss of heterozygosity at chromosome 18q.

as an important step in the development of many CRCs. Among the genes located on 18q are the *DCC* gene that codes for a neutrin-1 receptor important in apoptosis, cell adhesion, and tumor suppression. *SMAD-4* gene, which codes for a nuclear transcription factor in transforming growth factor-β1 signaling, is involved in tumor suppression and the *SMAD22* gene is involved in endodermal differentiation[44].

MSI is a measure of the inability of the DNA nucleotide mismatch repair system to correct errors that commonly occur during the replication of DNA. It is characterized by the accumulation of single nucleotide mutations and length alterations in repetitive microsatellite nucleotide sequences. It is an alternative pathway to chromosomal instability with LOH in the pathogenesis of colon cancer[11].

In conclusion, the results of this study support the opinion that in colon cancer, genetic approaches may delineate subgroups of patients who would benefit most from specific treatment or other investigative therapies. We found that 18qLOH is a very informative prognostic genetic marker that could lead to the identification of patients who should be subjected to different adjuvant therapy plans. In addition, our study demonstrated that MSI-H in colon cancer is a potentially favorable prognostic genetic marker. This lends support to the hypothesis that MSI-H is an index of a less aggressive type of tumor growth and a heightened immunologic response. Although these results suggest that 18qLOH and MSI are prognostic genetic markers, the final verdict should depend on large-scale prospective randomized control trials.

COMMENTS

Background

It has been shown that colorectal cancer (CRC) arises through at least two distinct genetic pathways of microsatellite alteration (MA): one involving chromosome instability presented as loss of heterozygosity (LOH), and the other involving microsatellite instability (MSI). Although many studies have proven the prognostic value of MA at chromosome 18q in CRC, the outcomes are controversial.

Research frontiers

To further define the clinical effect of molecular genetic alterations on clinicopathological features and prognosis, the authors analyzed 106 patients retrospectively with stage Ⅱ colon cancer for LOH and MSI at chromosome 18q.

Innovations and breakthroughs

In order to minimize the bias, the authors selected a relatively small range of tumor locations (colon cancer) and TNM stage (stage Ⅱ) to analyze expression of LOH and MSI focusing on five microsatellite loci at chromosome 18q.

Applications

This study found LOH at chromosome 18q to be a very informative prognostic genetic marker that could identify patients who should be subjected to different adjuvant therapy plans.

Peer review

The manuscript presented data of LOH and MSI assays on more than 100 cases of stage Ⅱ colonic adenocarcinomas. The results showed the preferential occurrence of allelic imbalance on 18q in more aggressive lesions, indicating the clinical significance of these markers.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

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Barcelona, Spain
18th United European Gastroenterology Week

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Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

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

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 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]

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

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

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

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

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

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Contents

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Contents

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EDITORIAL

Oxidative stress and antioxidants in hepatic pathogenesis

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Abstract

Long term hepatitis B virus (HBV) infection is a major risk factor in pathogenesis of chronic liver diseases, including hepatocellular carcinoma (HCC). The HBV encoded proteins, hepatitis B virus X protein and preS, appear to contribute importantly to the pathogenesis of HCC. Both are associated with oxidative stress, which can damage cellular molecules like lipids, proteins, and DNA during chronic infection. Chronic alcohol use is another important factor that contributes to oxidative stress in the liver. Previous studies reported that treatment with antioxidants, such as curcumin, silymarin, green tea, and vitamins C and E, can protect DNA from damage and regulate liver pathogenesis-related cascades by reducing reactive oxygen species. This review summarizes some of the relationships between oxidative stress and liver pathogenesis, focusing upon HBV and alcohol, and suggests antioxidant therapeutic approaches.

Key words: Hepatitis B virus; Hepatitis B virus X protein; Alcohol; Chronic liver disease; Oxidative stress; Antioxidant

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent tumor types worldwide. It is the fifth most common cancer and the third leading cause of cancer death[1]. There are multiple etiological agents that are associated with the development of HCC, the most frequent being chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, and long-term exposure to the mycotoxin, aflatoxin B1.

HBV is recognized as a major etiological factor in the development of such diseases as fatty liver (steatosis), cirrhosis, hepatocellular adenoma, and HCC[2,3]. The risk of HCC in chronic HBV carriers is more than 100 times greater than in uninfected individuals. In the year 2000, worldwide new cases of HCC had increased to 564 300[4]. More than 80% of these cases occur in developing countries, especially Southeast Asia and sub-Saharan Africa. Some 80%-90% of HCCs develop in cirrhotic liver[5]. After 20-30 years of chronic infection, 20%-30% of patients develop liver cirrhosis. HCC develops at an annual rate of 3%-8% in HBV-infected cirrhotic patients[6].

In the course of chronic infection, fragments of HBV

DNA integrate randomly into host DNA. Many of these integrated species encode the hepatitis B virus X protein (HBx) and truncated preS polypeptides, which contribute major steps in hepatocarcinogenesis. HBx binds to the DDB1 subunit of a UV-damaged DNA binding protein[7], the latter of which appears to be important for maintaining the integrity of DNA repair[8]. HBx has also been shown to bind to and functionally inactivate p53[9,10].

Therefore, the HBx and HBs proteins represent the two potential candidate proteins involved in HBV-related hepatocarcinogenesis[11-16]. HCC is also a common complication of alcoholic cirrhosis, although ethanol appears to not be directly carcinogenic[17].

OXIDATIVE STRESS

Oxidative stress is a disturbance in the oxidant-antioxidant balance leading to potential cellular damage. Most cells can tolerate a mild degree of oxidative stress, because they have sufficient antioxidant defense capacity and repair systems, which recognize and remove molecules damaged by oxidation. The imbalance can result from a lack of antioxidant capacity caused by disturbances in production and distribution, or by an overabundance of reactive oxygen species (ROS) from other factors. ROS are potential carcinogens because of their roles in mutagenesis, tumor promotion, and progression[18]. If not regulated properly, the excess ROS can damage lipids, protein or DNA, inhibiting normal function[19]. ROS alterations in different signaling pathways may modulate gene expression, cell adhesion, cell metabolism, cell cycle and cell death. These events may induce oxidative DNA damage, which in turn increases chromosomal aberrations associated with cell transformation[20]. ROS may also activate cellular signal pathways, such as those mediated by mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB), phosphatidylinositol 3-kinase (PI3K), p53, β-catenin/Wnt and associated with angiogenesis[21-23]. Importantly, HBx stimulates the activities of MAPK, NF-κB, PI3K, and β-catenin (as well as other pathways) that are thought to contribute importantly to the development of HCC. Perhaps this is why carriers with chronic liver disease (CLD) develop a high incidence of HCC, while asymptomatic carriers do not.

OXIDATIVE STRESS EFFECT ON CHRONIC LIVER DISEASE AND LIVER FIBROSIS

Several *in vitro* and *in vivo* observations suggest that oxidative stress and associated damage could represent a common link between different forms of chronic liver injury and hepatic fibrosis. For example, oxidative stress contributing to lipid peroxidation is one of the critical factors involved in the genesis and the progression of nonalcoholic steatohepatitis and liver cancer[24,25]. Viral infection or alcohol abuse greatly increased the highly variable miscoding etheno-modified DNA like epsilondA [1,N(6)-etheno-2'-deoxyadenosine] levels by triggering lipid peroxidation. Patients with chronic hepatitis, liver cirrhosis, and HCC due to HBV infection had more than 20 times higher urinary epsilondA levels[25] compared to uninfected individuals with no liver disease.

Among the mechanisms involved in mediating the process of liver fibrosis, an important role is played by ROS[26]. During the progression of liver injury, hepatic stellate cells (HSCs) become activated, which produce extracellular matrix such as collagen I [27]. Collagen I gene regulation has revealed a complex process involving ROS as a key mediator[28-30]. ROS-sensitive cytokines contribute to HSC activation during inflammation through paracrine signals released from immune cells[31]. The activated HSCs become responsive to platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β. PDGF facilitates the progression of hepatic fibrosis in human CLD. It increased the accumulation of hydrogen peroxide in HSCs. Specifically PDGF-induced increases in collagen deposition and liver fibrosis is markedly reduced by treatment with the anti-oxidant drug Mn-TBAP[32,33]. TGF-β increases ROS production and decreases the concentration of glutathione (GSH)[34]. In this context, it is important to note that HBx trans-activation activity is stimulated by ROS. Given that HBx is also associated with the development of HCC in both human carriers and in transgenic mice, and that HCC is associated with chronic inflammation, this underscores the importance of inflammation in the context of chronic HBV infection to hepatocarcinogenesis.

HBV INFECTION AND OXIDATIVE STRESS

Many groups have shown that HBV can induce oxidative stress using HBV transgenic mice or HBV DNA transfection of cells *in vitro*, while oxidative stress is also common among HBV infected patients with CLD[35-41]. Oxidative stress also precedes the development of HCC in transgenic mice that overproduce and accumulate intracellular HBsAg. Several studies have found that the total peroxide level, a parameter of oxidative stress, is significantly higher in patients with chronic hepatitis compared to asymptomatic carriers, and positively correlated with alanine aminotransferase (ALT) levels, suggesting that oxidative stress plays a critical role in hepatic injury. Oxidative stress is also associated with the severity of the disease. Lipid peroxidation and oxidative DNA damage are enhanced in patients with HBV infection.

Mitochondria are a major source of ROS. ROS can form through electron leakage from the mitochondrial respiratory chain[42]. HBx itself targets mitochondria and directly interacts with voltage-dependent anion channel 3. It alters the mitochondrial membrane potential and increases the endogenous ROS level[43-46]. HBx expression also induces oxidative stress through calcium signaling and activates cellular kinases, leading to the activation of transcription factors NF-κB, signal transducer and activator of transcription 3, and others *via* phosphorylation[47,48]. It is observed that HBV-induced oxidative stress also stimu-

lates the translocation of mitogen-activated protein kinase Raf-1 to mitochondria. This activation involves both the Src- and the PAK-mediated phosphorylation of the Raf-1 activation domain[49]. HBx also induces lipid peroxidation *via* down-regulation of SeP expression, resulting in increased expression of tumor necrosis factor-α in the human hepatoblastoma cell line, HepG2[50].

Activity of the anti-oxidant enzymes CuZn-SOD and GSH-Px was found to be the lowest in chronically infected patients compared with other groups[51,52]. Detection of an increase in MDA levels, which is a product of lipid peroxidation in HBV infected groups, indicates that oxidative stress is increased in HBV infection[52,53]. After treatment with interferon-α and lamivudine, however, there was a decrease in the products of lipid peroxidation and an increase in the antioxidant enzymes, such as CuZn-SOD and GSH-Px, compared with pretreatment[53].

The marker 8-hydroxydeoxyguanosine (8-OHdG) is useful in estimating DNA damage induced by oxidative stress. Importantly, hepatic 8-OHdG accumulation was detected in patients with chronic hepatitis B[39,54]. Further, HBV replication causes oxidative stress in HepAD38 liver cells, with more than 3 fold increases in the GSSG/GSHtot ratio[37].

HuH-7 cells carrying the pre-S mutant (a truncated form of preS/S polypeptide) exhibited enhanced levels of ROS and oxidative DNA damage through endoplasmic reticulum (ER) stress pathways. Oxidative DNA damage has also been observed in livers of transgenic mice carrying the pre-S mutant[36]. HepG2-HBx cells and the livers of HBx mice also showed increased ROS levels (Figure 1), mtDNA deletion, and declines in the mitochondrial membrane potential compared to controls (data not shown). Through DNA chip analysis, several ROS-related molecules, such as members of the CYP450 families, were altered in HBx transgenic mice. The cytochrome p450s are a superfamily of hemeproteins that serve as terminal oxidases[55]. A major function of these p450s is to convert compounds into more polar metabolites[56]. Detoxification by cytochrome p450 can also produce ROS[57,58]. CYP2E1, a member of the p450 family that oxidizes ethanol, generates oxidative stress in the mitochondrial compartment of hepatocytes. This has been suggested to play a role in hepatotoxicity, as observed in ALD-related patients[59-61]. In a mouse model of nonalcoholic steatosis, CYP2E1 also plays key roles in ROS production and contributes to the pathogenesis of liver damage[62,63]. Thus, the involvement of mitochondria in the production of free radicals resulting from ethanol metabolism, and the fact that elevated free radical formation stimulates HBx activities, combined with the ability of mitochondria to oxidize ethanol may help to explain the apparent synergistic effects of chronic ethanol intake and HBx expression on the pathogenesis of CLD and HCC.

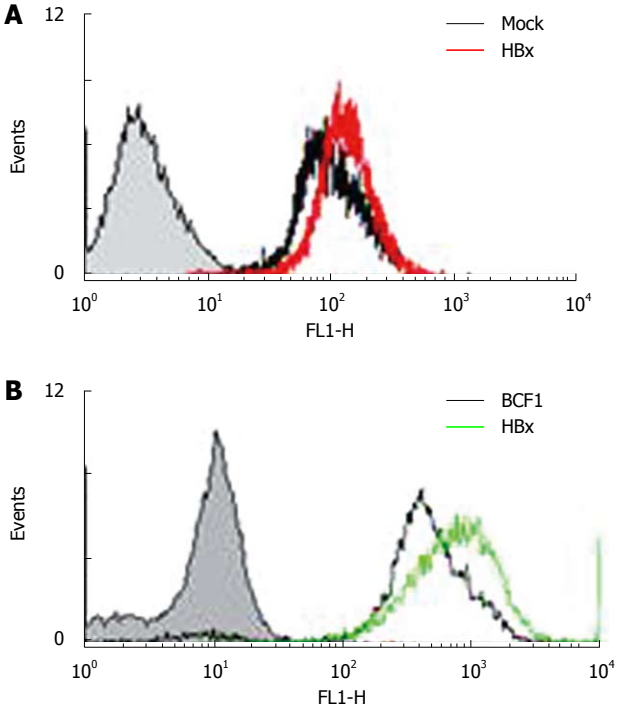

Figure 1 Increased reactive oxygen species in hepatitis B virus X protein transfected HepG2 stable cell line and hepatitis B virus X protein transgenic mouse hepatocytes. Reactive oxygen species (ROS) was detected by FACS caliber using dichlorofluorescein diacetate (DCFA-DA). A: HepG2 cell line stably transfected with hepatitis B virus X protein (HBx) showed a higher level of ROS compared to control cells; B: ROS production was checked after 4 wk of male HBx and control mouse hepatocyte growth. HBx mice hepatocytes generate more ROS than control mice.

LIVER PATHOGENESIS BY ALCOHOL-INDUCED OXIDATIVE STRESS

Chronic alcohol consumption has long been associated with progressive liver disease[64,65]. The liver is the major site of ethanol metabolism and thus sustains the most injury from chronic alcohol consumption. In alcohol-related liver disease, free radicals play a part in the pathogenesis of liver damage. Acute and chronic ethanol treatment increases ROS production, lowers cellular antioxidant levels, and enhances oxidative stress in many tissues, especially the liver[66,67]. It induces an accumulation of cysteine, a glutathione precursor/metabolite in the liver, probably due to gamma-glutamyltransferase induction[68]. Acetaldehyde produced by the oxidation of alcohol is able to inhibit the repair of alkylated nucleoproteins, to decrease the activity of several enzymes, and to damage mitochondria. Acetaldehyde also promotes cell death by depleting the concentration of reduced glutathione, by inducing lipid peroxidation, and by increasing the toxic effects of free radicals. Finally, acetaldehyde has been shown to directly stimulate proliferation of HSC and to increase collagen synthesis[69-71].

Chronic ethanol treatment has long been known to depress mitochondrial function[72-74]. The occurrence of DNA fragmentation in peripheral blood lymphocytes reflects a direct genotoxic effect of alcohol, HBV, and/or HCV, and suggests that the same genotoxic effect may operate in the liver and contribute to hepatocarcinogenesis[75].

Alcohol is also metabolized by mitochondrial CYP2E1. Ethanol exposure to VL-17A cells increased CYP2E1, decreased the activity of antigen-trimming enzymes

Table 1 Serum glutamate oxalate-transferase and glutamate-pyruvate-transferase values of wild and Hepatitis B virus X protein mice

Groups	Age (mo)	No. of animals	Treatment	Duration (wk)	GOT (U/L)	GPT (U/L)
HBx-tg	8	8	25% alcohol	12	193 ± 83.5	87.3 ± 35.5
	8	4	Normal water	12	60 ± 13.8	82 ± 19
C57BL/6J	8	8	25% alcohol	12	119 ± 31.9	61.7 ± 11.5
	8	9	Normal water	12	42 ± 11	68 ± 6

GOT: Glutamate oxalate-transferase; GPT: Glutamate-pyruvate-transferase; HBx: Hepatitis B virus X protein.

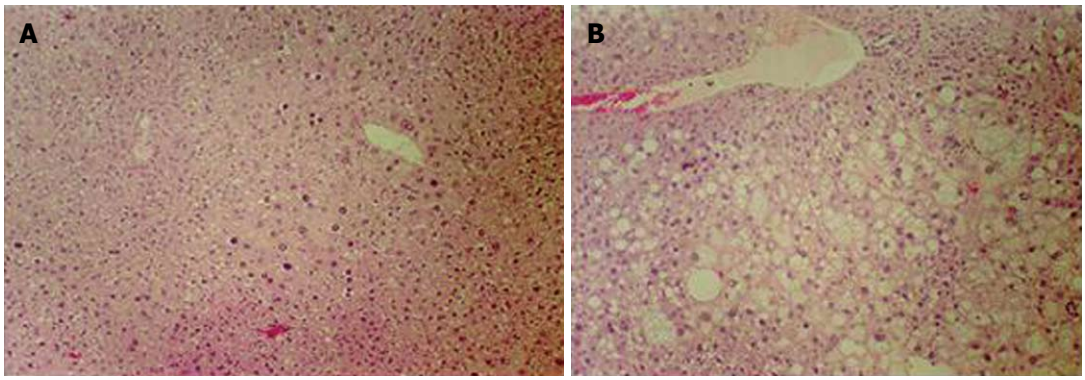

Figure 2 Chronic ethanol consumption caused liver damage in hepatitis B virus X protein transgenic mice. Ethanol fed hepatitis B virus X protein (HBx) tg mouse liver (B) showed severe liver damage, hepatocyte enlargement and fatty changes compared with water fed HBx (A). Original magnifications 100 ×.

like proteasome peptidase and leucine aminopeptidase (LAP). This defect may potentially result in decreased MHC class I -restricted antigen presentation on virally infected liver cells[68].

Alcohol-induced inflammatory and innate immune responses in Kupffer cells, due to elevated gut-derived plasma endotoxin levels, increase ROS-induced damage, and profibrogenic factors such as acetaldehyde or lipid peroxidation products, contribute to activation of HSCs[76]. Following a fibrogenic stimulus such as alcohol, HSCs transform into activated collagen-producing cells. There is much current interest in the likely synergistic interactions between hepatitis viruses and alcohol, especially with respect to generating oxidative stress.

Alcohol exacerbates pathological changes in HBx transgenic mice

C57BL/6J (control) and HBx transgenic mice 8 mo of age were fed with water or 25% ethanol liquid diets for 12 wk (Table 1). Glutamate oxalate-transferase (GOT) and glutamate-pyruvate-transferase (GPT) levels, both indicators of liver damage, were elevated in control and HBx ethanol-fed groups, but not in the water-fed groups. However, HBx mice showed higher levels of GPT (87.3 ± 35.5 U/L) and GOT (193 ± 83.5 U/L) than wildtype mice (GPT: 61.7 ± 11.5 U/L, GOT: 119 ± 31.9 U/L). This result indicated that HBx transgenic mice developed more severe liver damage from ethanol than control mice. This was confirmed by histological evaluation of the liver, which showed the development of more severe liver injury only in the HBx transgenic mice. Hyperplastic nodules, found in both the water- and ethanol-fed groups of HBx transgenic mice, were more frequent among the ethanol-treated group (Figure 2). Control mice fed ethanol showed mild steatosis (data not shown), but the alcohol-treated HBx transgenic liver had severe steatosis and hepatomegaly compared to the untreated controls (Figure 2). Thus, even moderate ethanol consumption promoted oxidative stress and liver injury in HBx transgenic mice, implying that compromised antioxidant defense promotes alcohol liver injury.

ANTIOXIDANT ENZYMES AND THE REDUCTION OF OXIDATIVE STRESS

Given that ROS production is a natural process, and that persistent, high levels of ROS could be damaging, the human body has developed antioxidant systems aimed at their neutralization. A variety of enzymatic and nonenzymatic mechanisms have evolved to protect cells against ROS. These include superoxide dismutase (SOD), which detoxifies the superoxide ion, catalase and the GSH peroxidase system, peroxiredoxins, which inactivate hydrogen peroxide (H_2O_2), and glutathione peroxidase, whose function is to detoxify cellular peroxides. Further, ceruloplasmin and ferritin help remove metals, such as iron, that promote oxidative reactions. There are also nonenzymatic, low-molecular-weight antioxidants, such as GSH, vitamin E, ascorbate (vitamin C), vitamin A, ubiquinone, uric acid, and bilirubin[77,78].

A CuZn-SOD is present in the cytosol and in the space between the inner and outer mitochondrial membranes, while a manganese-containing SOD is present in the mitochondrial matrix. Both of these enzymes are critical for prevention of ROS-induced toxicity[79].

Catalase is found primarily in peroxisomes; it catalyzes a reaction between two H_2O_2 molecules, resulting in the

formation of water and O_2. In addition, catalase can promote the interaction of H_2O_2 with hydrogen donors so that the H_2O_2 can be converted to one molecule of water, and the reduced donor becomes oxidized (peroxidatic activity of catalase).

The Prx family has the capacity to decompose H_2O_2 *in vivo* and *in vitro*. All Prx enzymes contain a conserved Cys residue that undergoes a cycle of peroxide-dependent oxidation and thiol-dependent reduction during catalysis. Mammalian cells express six isoforms of Prx (Prx I to VI), which are classified into three subgroups (2-Cys, atypical 2-Cys, and 1-Cys) based on the number and position of Cys residues that participate in catalysis. Prx I to Prx IV are members of the 2-Cys Prx subgroup. Prx I and Prx II exist in the cytosol. Prx III, which is synthesized with a mitochondrial targeting sequence, is imported into and matures within mitochondria. Prx IV is a secreted protein[80-83]. Prx V is expressed ubiquitously; it localizes to mitochondria and peroxisomes[84] and possesses antioxidant activity equivalent to that of catalase[85]. All peroxiredoxins have two cysteine residues, but Prx VI has only one at position 47. Prx VI is the only peroxiredoxin whose target is glutathione rather than thioredoxin. It is mostly cytosolic.

ANTIOXIDANT THERAPY FOR CHRONIC LIVER DISEASE

As discussed above, oxidative stress plays a central role in HBV- and alcohol-induced liver damage. There are several possible strategies for preventing this stress[34]. Among them is the addition of antioxidant agents to antiviral drugs for patients with chronic hepatitis B.

Curcuminoids

For example, curcuminoids, the main yellow pigments in Curcuma longa (turmeric), have been used widely and for a long time in the treatment of sprains and inflammation[86]. Curcumin is the main component of turmeric, and two minor components are also present as curcuminoids. Curcuminoids possess antioxidant activity[87]. They protect DNA against oxidative attack, thereby lowering the risk for mutations and other genetic damage[88,89]. They also activate detoxification enzymes such as glutathione S-transferase[90]. Curcumins can down-regulate NF-κB, a nuclear transcription factor and critical upstream regulator of genes that control acute and chronic inflammation cascades[91,92]. Curcumin exerts beneficial effects in animal models of liver injury and cirrhosis[93,94]. Curcumin prevents alcohol-induced liver disease in rats by blocking activation of NF-κB[95] and by induction of HO-1[96]. Curcumin inhibits the fibrogenic progression of murine steatohepatitis[97]. It inhibits extracellular matrix formation by enhancing HSC matrix metalloproteinase expression *via* $PPAR_\gamma$ and suppresses connective tissue growth factor expression[98]. CLL extract also represses HBV replication by enhancing the level of p53 protein[99].

Silymarin

Silymarin is a purified extract from milk thistle [*Silybum marianum* (L.) Gaertn], composed of a mixture of four isomeric flavonolignans: silibinin (its main, active component), isosilibinin, silydianin, and silychristin. This extract has been used as a remedy for almost 2000 years[100] and continues to be used as a medicine for many types of acute and chronic liver diseases. Silybin is an effective antioxidant, conserving GSH in liver cells while stabilizing the liver cell membranes against oxidative attack[100,101].

Inhibition of liver fibrogenesis in clinical trials, and promotion of liver regeneration[102,103] have been inconsistent with these treatments. In clinical trials among patients with viral hepatitis[104], alcoholic liver damage[105], and/or other liver diseases, silymarin and silybin lowered liver enzymes and (at times) improved antioxidant status, but did not consistently improve symptoms[104,105]. It is routinely used in the clinic as a hepatoprotectant. Silymarin exerts beneficial effects on the early stages of chronic liver disease, preventing and delaying the onset of HBV-related liver carcinogenesis[106-110].

Mechanistically, the anti-inflammatory and anticancer effects of silybin and the other flavonolignans are related to the potent inhibition of NF-κB. Silybin is a potent inhibitor of NF-κB activation, as induced by a variety of anti-inflammatory agents[111].

Green tea

Green tea, a product of the plant Camellia sinensis (family Theaceae), contains polyphenols, specifically catechins of the flavan-3-ol class and their gallate derivatives. They are potent antioxidant and anti-inflammatory agents[112]. The flavan-3-ol structure makes them efficient scavengers of superoxide, singlet oxygen, nitric oxide, and peroxynitrite[113]. They up-regulate antioxidant and other detoxifying enzymes and protect DNA from oxidative damage[114-116]. Like other flavonoids, the green tea catechins can down-regulate NF-κB and AP-1, both of which may promote chronic inflammation and carcinogenesis when abnormally activated[117].

When treated with natural green tea extract, cells supporting HBV replication had reduced virus gene expression and reduced cell growth[118].

Vitamins C and E

Vitamin C is essential to a healthy diet as well as a highly effective antioxidant. It is a substrate for ascorbate peroxidase. Vitamin E is a fat-soluble antioxidant that is the major antioxidant found in lipid-phase membranes. It blocks the production of ROS formed when fat undergoes oxidation[119]. Several studies have clearly shown that serum levels of vitamin E are significantly reduced in patients with alcoholic liver disease[120,121]. Vitamin E levels also negatively correlate with production of oxidative stress products and directly correlate with the extent of liver damage[122]. Therefore, maintenance of normal concentrations of vitamin E seems to be essential to prevent lipid peroxidation induced by alcohol consumption. Works from several laboratories have indicated that mitochondrial damage may present a common early event in cell injury[123]. Mitochondrial damage was prevented by vitamin E[124]. Vitamin E or C alone or in combination can facilitate scavenging free

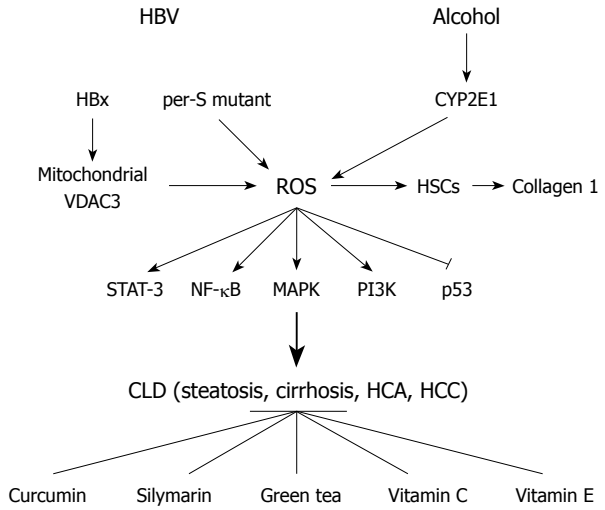

Figure 3 Summary of hepatitis B virus and alcohol induced reactive oxygen species effects on chronic liver disease and antioxidant's protective effects. HBV: Hepatitis B virus; HBx: Hepatitis B virus X protein; VDAC3: Voltage-dependent anion channel 3; ROS: Reactive oxygen species; HSCs: Hepatic stellate cells; STAT-3: Transducer and activator of transcription 3; NF-κB: Nuclear factor κB; MAPK: Mitogen-activated protein kinase; PI3K: Phosphatidylinositol 3-kinase; CLD: Chronic liver disease; HCA: Hepatocellular adenoma; HCC: Hepatocellular carcinoma.

radicals generated in liver tissue[125]. Pretreatment with vitamin C against imidacloprid-induced oxidative liver stress in mice is better than post-treatment administration[126]. Pretreatment with vitamin E reduced the degree of oxidative stress[90], although this vitamin produced only slight changes in hepatic injury[127]. In the mouse model, vitamin E supplementation restored alcohol-induced redox status, reduced apoptosis, and prevented oxidative stress[128]. In addition, vitamin E in doses of 600 mg daily was effective in suppressing HBV replication and normalizing ALT in a significant proportion of chronically infected patients with CLD[129]. In this context, it will be important to determine whether anti-oxidants reduce HBxAg expression and/or function in cultured cells, or promote the resolution of CLD in human carriers and/or among human carriers with CLD who are also chronic alcoholics. If so, then anti-oxidant treatments may reduce the risk for progressive CLD lesions ultimately resulting in HCC, and/or eliminate the synergy between HBV and chronic alcoholism in the pathogenesis of alcoholic liver disease.

CONCLUSION

In summary (Figure 3), HBV and alcohol-induced liver injury are multi-step processes involving several mechanisms. The ability of HBV and alcohol to induce oxidative stress and the role of ROS in HBV- or alcohol-triggered liver damage is an important area of research, particularly because that information could be of major therapeutic value in protecting the liver. As basic information continues to emerge regarding the role of oxidative stress in disease development and the mechanisms underlying ROS-related cellular toxicity, these findings will lead to more rational antioxidant therapeutic approaches.

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EDITORIAL

Hepatic organ protection: From basic science to clinical practice

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Abstract

Hepatic ischemia and reperfusion (I/R) injury during liver surgery is still the main cause of postoperative liver failure and the subsequent rise of mortality in these patients. During the last few years, a multitude of underlying mechanisms have been extensively characterized and many different protective approaches have been evaluated under experimental conditions. Some of them have already found their way into small sized clinical trials. In this Topic Highlight series of articles, we present recent insights into promising protective concepts including the regulation and optimization of hepatic blood flow, molecular mechanisms of preconditioning and pharmacological approaches with the aim of limiting hepatic I/R injury. Leading international experts present the latest experimental evidence in their fields stressing clinically relevant ideas, which are now on the edge of entering clinical practice.

Key words: Heme oxygenase-1; Hepatectomy; Hepatic organ protection; Ischemia/reperfusion injury; Liver blood flow; Liver transplantation; Preconditioning

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The sequence of hepatic ischemia and reperfusion (I/R) is frequently associated with the destruction of liver cells thus contributing to postoperative liver failure and increased mortality. In liver transplantation, up to 30% of delayed graft function are caused by the consequences of hepatic I/R injury[1]. Intermittent clamping of hepatic blood inflow (e.g. Pringle's maneuver), an established technique to reduce blood loss during major liver resection, could have similar adverse effects by I/R mediated mechanisms. Furthermore, van der Bilt and colleagues demonstrated that I/R induced by vascular clamping is a strong stimulus that promotes the outgrowth of micrometastasis in the liver[2]. Therefore, the development of therapeutic concepts to prevent hepatic I/R injury has become the focus of intensive research efforts during the last few years. In this Topic Highlight series, we have put together a group of international experts providing an update on the latest achievements in their fields stressing clinically relevant ideas with the aim of protecting the liver against I/R injury. The maintenance of macro- and microvascular perfusion after hepatic ischemia plays a crucial role in the prevention of liver cell injury. In the first article of the present review series, Eipel *et al*[3] discuss recent insights into the regulation of hepatic blood flow and in particular the relevance of the "hepatic arterial buffer response", an important intrinsic mechanism of the hepatic artery to produce compensatory flow changes in response to changes in portal venous flow. The authors present detailed experimental and clinical information stressing the crucial importance of the hepatic arterial buffer response

as a regulatory mechanism to maintain adequate liver function and metabolic homeostasis. The second contribution focuses on preconditioning, an important phenomenon mediating cytoprotection in many different organs including the liver. Alchera *et al*[4] provide an interesting overview on the molecular mechanisms of liver preconditioning with special emphasis on the development of pharmacological approaches aimed at activating intrinsic protective systems in patients undergoing liver surgery. The next review concentrates on one of the most powerful inducible enzymes known today: heme oxygenase-1 (HO-1). HO-1 metabolizes heme into iron, carbon monoxide, and biliverdin, which is subsequently converted to bilirubin. Upregulation of HO-1 and administration of each of its reaction products has been shown to play a pivotal role in the maintenance of cellular function after sublethal stress in nearly all organ systems including the liver. However, the development of therapeutic strategies that utilize the protective effect of HO-1 induction is hampered by the fact that most pharmacological inducers of this enzyme perturb organ function by themselves and that gene therapy for upregulation of HO-1 has potential negative side effects, which currently preclude its clinical application under these conditions. Hence, most substances used for upregulation of HO-1 under experimental conditions are not available for use in patients because of their toxicity and undesirable or unknown side effects[5]. During the last years, a few non-toxic HO-1 inducing compounds have been identified in animal experiments including the β_1-agonist dobutamine, the phosphodiesterase-Ⅲ-inhibitor olprinone, and the volatile anesthetics isoflurane and sevoflurane. Isoflurane has been shown to profoundly protect the liver against I/R injury by upregulation of HO-1 gene expression under experimental conditions[6,7]. As a consequence, volatile anesthetics are currently being evaluated for their potential to induce HO-1 and protect the liver against I/R in humans. In the present series of reviews, Richards *et al*[8] summarize HO-1 mediated protective effects within the liver and point to its therapeutic potential in detail. The protective role of nitric oxide (NO) in the context of hepatic I/R injury is then nicely presented by Siriussawakul and coworkers in their contribution[9]. The authors discuss the influence of endogenous NO on hepatic I/R injury and the potential therapeutic role of inhaled NO, nitrite and other NO donors in ameliorating hepatic I/R injury. Next, Mathes systematically describes the current knowledge on the antioxidant and other protective actions of melatonin in the liver. Melatonin, the "hormone of darkness", has recently been shown to exert abundant hepatoprotective effects in a multitude of experimental studies. Mathes illustrates this topic in depth and highlights possible approaches for its beneficial use in patients[10]. Finally, Gurusamy *et al*[11] present the currently available clinical data concerning protective strategies in liver surgery and review the significance of these studies in an evidence-based approach.

The present Topic Highlight series "Hepatic organ protection: From basic science to clinical practice" is far from being a complete reference of all experimental evidence concerning liver protection. It presents fascinating clinically relevant experimental concepts aimed at the identification of surgical techniques and pharmacological compounds, which now have to be validated in large randomized clinical trials.

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TOPIC HIGHLIGHT

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Regulation of hepatic blood flow: The hepatic arterial buffer response revisited

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Abstract

The interest in the liver dates back to ancient times when it was considered to be the seat of life processes. The liver is indeed essential to life, not only due to its complex functions in biosynthesis, metabolism and clearance, but also its dramatic role as the blood volume reservoir. Among parenchymal organs, blood flow to the liver is unique due to the dual supply from the portal vein and the hepatic artery. Knowledge of the mutual communication of both the hepatic artery and the portal vein is essential to understand hepatic physiology and pathophysiology. To distinguish the individual importance of each of these inflows in normal and abnormal states is still a challenging task and the subject of ongoing research. A central mechanism that controls and allows constancy of hepatic blood flow is the hepatic arterial buffer response. The current paper reviews the relevance of this intimate hepatic blood flow regulatory system in health and disease. We exclusively focus on the endogenous interrelationship between the hepatic arterial and portal venous inflow circuits in liver resection and transplantation, as well as inflammatory and chronic liver diseases. We do not consider the hepatic microvascular anatomy, as this has been the subject of another recent review.

PHYSIOLOGY OF LIVER BLOOD FLOW AND HEPATIC MACROHEMODYNAMICS

Hepatic blood flow and hepatic pressures

The liver has the most complicated circulation of any organ. According to the anatomical peculiarity of the double afferent blood supply of the liver, 75%-80% of the blood entering the liver is partially deoxygenated venous blood supplied by the portal vein, which collects all the blood that leaves the spleen, stomach, small and large intestine, gallbladder and pancreas[1-3]. The hepatic artery accounts for the remaining 25% with well-oxygenated blood. Total hepatic blood flow ranges between 800 and 1200 mL/min, which is equivalent to approximately 100 mL/min per 100 g liver wet weight[4]. Although the liver mass constitutes only 2.5% of the total body weight, the liver receives nearly 25% of the cardiac output.

The valveless portal vein is a low pressure/low resistance circuit, while the hepatic artery supplies the liver with arterial blood in a high pressure/high resistance system[4]. The mean pressure in the hepatic artery is similar to that in the aorta, while portal vein pressure has been reported

to range between 6 and 10 mmHg in humans when determined by direct cannulation[5] or by splenic puncture[6]. Portal pressure depends primarily on the degree of constriction or dilatation of the mesenteric and splanchnic arterioles and on intrahepatic resistance. Both afferent systems merge at the sinusoidal bed, where the pressure is estimated to be slightly, namely, 2-4 mmHg above that in the smallest collecting veins or the inferior vena cava.

Hepatic blood volume

Although only limited data exist, it appears that hepatic blood volume ranges from 25 to 30 mL/100 g liver weight, and accounts for 10%-15% of the total blood volume[7]. Estimations of hepatic blood volume are highly variable, as indirect calculations of hepatic blood volume from red blood cell content of the liver and arterial hematocrit are inaccurate, and hepatic venous pressure largely influences hepatic blood volume[4]. Furthermore, rough estimation suggests that more than 40% of the hepatic blood is held in large capacitance vessels (portal vein, hepatic artery and hepatic veins), while the sinusoids accommodate up to 60% as small vessel content[4]. Of note is the high compliance of the hepatic vascular bed, calculated as the change in blood volume per unit change in venous pressure[8]. In cats, the hepatic blood volume increases in response to elevated venous pressure and is doubled when hepatic venous pressure is elevated to 9.4 mmHg[8]. Hepatic blood volume may expand considerably in cardiac failure and, in turn, serves as an important blood reservoir in case of bleeding episodes, and compensates up to 25% of the hemorrhage by immediate expulsion of blood from the capacitance vessels[9].

Hepatic oxygen consumption

As in any other artery of the body, oxygen saturation of the hepatic artery usually exceeds 95%. Oxygen saturation of portal blood during the fasting state ranges up to 85%, which is greater than that of other systemic veins; however, it substantially drops after food ingestion. It is generally accepted that 50% of the oxygen requirements of the liver are provided by portal venous blood and the other half derives from the hepatic artery[1]. If oxygen demand is increased, the liver simply extracts more oxygen from the blood in order to maintain oxygen uptake. In line with this, alterations of hepatic oxygen supply, attained by isovolemic hemodilution or stimulation of hepatic enzymes, lead to reduced oxygen content in the inflow and outflow vessels, but do not cause dilatation of the hepatic artery, which disproves the view that the hepatic artery might be regulated by the metabolic activity of the liver cell mass[10].

Hepatic blood flow control

Liver blood flow is controlled by mechanisms that are independent of extrinsic innervation or vasoactive agents that regulate (1) hepatic arterial inflow; (2) portal venous inflow; and (3) the interrelationship between hepatic arterial and portal venous inflow circuits. The relationship between arterial pressure and hepatic arterial blood flow has been analyzed in several species. However, there is disagreement as to whether the hepatic arterial vasculature exhibits autoregulation of blood flow. The term autoregulation is specifically used to describe the non-linearity of the arterial pressure-to-arterial flow relationship and comprises the tendency for local blood flow to remain constant in the face of pressure changes in the arteries that perfuse a given organ. Some studies have revealed evidence of pressure-dependent autoregulation of blood flow in the hepatic arterial bed[11-15]. In denervated dog liver preparations, Hanson and Johnson have shown that a step-wise reduction of hepatic artery pressure from 90 to 30 mmHg was accompanied by a substantial reduction in hepatic artery resistance[15]. Comparably, livers with intact peri-arterial nerve plexi showed a 60% decrease in arterial resistance upon a 63% pressure reduction[11]. Overall, however, the degree of autoregulation is considered small[11] and present in only about 60% of all preparations[15]. The fact that papaverine infusion can abolish hepatic artery dilatation indicates that the observed effects are primarily mediated by myogenic adaptation of the vasculature to changes in transmural pressure[11]. Besides that, a metabolic washout hypothesis is also tenable, where the hepatic artery washes out the endogenous adenosine, thereby completely accounting for autoregulation of the hepatic artery[16].

Less controversy exists concerning pressure-to-flow autoregulation of the portal venous vascular bed. Only a few studies have indicated autoregulation of portal venous blood flow[13], while the majority of studies have revealed a linear pressure-to-flow relationship with constant or increased portal venous resistance at low pressure gradients. In fact, there is even evidence for an opposite effect with (1) a partial passive collapse of the portal vascular bed taking place upon reduction of portal pressure; and (2) a reciprocal decrease in resistance upon a step-wise increase in portal venous pressure[15].

REGULATION OF LIVER BLOOD FLOW BY THE HEPATIC ARTERIAL BUFFER RESPONSE

Besides the intrinsic regulation of the hepatic artery by the classical arterial autoregulation, that is, the myogenic constrictive response of the hepatic artery if the arterial pressure rises, there is a second form of intrinsic regulation, termed the hepatic arterial buffer response (HABR). This unique mechanism represents the ability of the hepatic artery to produce compensatory flow changes in response to changes in portal venous flow. Although Burton-Opitz observed an increase in hepatic arterial blood flow upon reduced portal venous inflow in 1911[17], this intimate relationship between these two vascular systems was termed HABR for the first time in 1981 by Lautt[18]. If portal blood flow is reduced, the hepatic artery dilates, and the hepatic artery constricts, if portal flow is increased[19,20]. Using transit-time ultrasonic volume flowmetry, intraoperative

measurement of the hepatic artery and portal venous flows in anesthetized patients with carcinoma of the splanchnic area has revealed a sharp and significant increase in hepatic arterial flow of about 30% after temporary occlusion of the portal vein, while temporary occlusion of the hepatic artery did not have any significant effect on portal venous circulation[20]. The HABR seems to operate in each individual under physiological conditions regardless of age. In addition, by establishing a method for measuring fetal hepatic arterial blood velocity, it has been reported that HABR even operates prenatally[21].

The increase in hepatic arterial blood flow is capable of buffering 25%-60% of the decreased portal flow[22,23]. The physiological role of this response is to minimize the influence of portal venous flow changes on hepatic clearance and to maintain adequate oxygen supply to tissues[24]. The latter function, however, may be of minor importance, since the liver normally receives more oxygen than it requires, and it can extract more oxygen to compensate for reduced delivery[25]. Thus, metabolic activity of the hepatic parenchymal cells does not directly control the hepatic arterial flow[22,25]. Instead, hepatic arterial flow subserves the hepatic role as a regulator of blood levels of nutrients and hormones by maintaining blood flow and thereby hepatic clearance as steadily as possible[24,26]. Because the portal vein cannot control its blood flow, which is simply the sum of outflows of the extrahepatic splanchnic organs, there is no reciprocity of the HABR, that is, alterations of the hepatic arterial perfusion do not induce compensatory changes of the portal vascular flow[18,20] or resistance[27].

The current view is that the HABR can be accounted for by the adenosine washout hypothesis[23]. This hypothesis states that adenosine is released at a constant rate into fluid in the space of Mall that surrounds the hepatic resistance vessels and portal venules. The space of Mall is contained within a limiting plate that separates this space from other fluid compartments. The concentration of adenosine is regulated by washout into the portal vein and the hepatic artery. If portal blood flow is reduced, less adenosine is washed away from the space of Mall, and the elevation in adenosine levels leads to dilation of the hepatic artery with a subsequent increase in hepatic arterial flow[10].

There are several lines of evidence that adenosine mediates the HABR: (1) adenosine produces hepatic arterial dilation[23]; (2) portal venous application of adenosine exerts one-half to one-third the effect of the same dose infused directly into the hepatic artery, which indicates that portal blood has some access to the arterial resistance vessels[10]; (3) adenosine uptake antagonists potentiate the HABR[23]; and (4) pharmacological antagonists of adenosine produce competitive blockade of the buffer response[16,28-30]. However, it has been suggested that adenosine itself does not diffuse from the portal venous to hepatic arterial bed to elicit the arterial response[31,32]. Rather ATP is released from the portal venous vasculature as a response to hypoxia associated with portal flow reduction, and diffuses into the hepatic arterial vasculature. No difference in the degree of inhibition of HABR by an adenosine antagonist has been observed between intra-arterial and intraportal injection of ATP in a rabbit model. This suggests that only the adenosine produced from ATP catabolism in the hepatic arterial vasculature contributes to arterial dilation. The adenosine produced from ATP in the portal venous vasculature is taken up effectively by the endothelium and vascular smooth muscle cells as soon as it is formed, and it does not diffuse to the hepatic arterial vasculature[30]. Mathie and Alexander have pointed out that adenosine is unlikely to be the sole regulator of HABR[33]. Other vasoactive compounds, such as nitric oxide and carbon monoxide, may be potential candidates to affect hepatic arterial flow and contribute to the HABR. Nitric oxide participates in regulation of hepatic arterial blood flow with changes in portal venous blood flow *via* ATP-dependent stimulation of endothelial purinergic receptors in the hepatic artery, which results in vasodilation[30,34,35]. Although nitric oxide is an important regulator of hepatic arterial resistance[36], it does not mediate the HABR and it is not found to play any significant role in total hepatic capacitance regulation[37].

Although nitric oxide serves as a potent vasodilator in the hepatic arterial circulation and exerts only a minor vasodilatory effect in the portal venous vascular bed, carbon monoxide is reported to maintain portal venous vascular tone in a relaxed state and to exert no vasodilation in the hepatic artery[38]. Recently, a third gaseous mediator, H_2S, has been recognized as an important endogenous vasodilator and neuromodulator[39]. There is now major evidence that H_2S also contributes to the HABR and partly mediates the vasodilatory response of the hepatic artery. This conclusion is based on the fact that supplementation of H_2S increases hepatic arterial conductance and almost doubles the buffer capacity. In turn, inhibition of the H_2S function by application of a selective inhibitor of K_{ATP} channels, which mainly mediate the ability of H_2S to relax vascular smooth muscle cells, markedly decreases buffer capacity[40].

Next to vasoactive mediators, there is evidence that sensory innervation and sensory neuropeptides are, at least to some extent, involved in the HABR. Accordingly, sensory denervated rats[41] and pigs[42] have revealed a reduced HABR upon partial occlusion of the portal vein. Furthermore, pretreatment with antagonists of calcitonin gene-related peptide (CGRP) and neurokinin (NK)-1 receptors significantly reduce the hepatic arterial blood flow, which indicates that the observed vasodilation in the vascular bed of the hepatic artery is due to stimulation of CGRP and NK-1 receptors[41].

IMPLICATIONS OF THE HABR IN LIVER DISEASES

HABR in liver resection, transplantation and laparoscopic surgery

The ability of the liver to regenerate after major resection has been studied extensively, but the factors responsible

for regeneration are not fully understood[43]. Although a clear association between flow and regenerative response has been suggested, the exact role of hepatic blood flow in liver regeneration is still a matter of intense debate. The increased blood flow to liver mass ratio immediately after partial hepatectomy (pHx) and the resultant increased intrahepatic shear stress have been proposed to stimulate and regulate liver regeneration[44-47]. On the other hand, the failure of the liver to control directly the portal venous blood flow has the consequence of portal hyperperfusion of the reduced-size liver (Figure 1A and B), which has been shown to impair seriously postoperative recovery of patients who are undergoing living donor liver transplantation or extended pHx[48,49]. In humans, 60% pHx results in a doubling of the portal flow in the 40% of remnant liver tissue[50]. This extent of pHx is followed by a transient and minor degree of small-for-size syndrome that usually resolves spontaneously within a few days. In contrast, major liver resection (> 75%) is followed by a more pronounced and long lasting small-for-size syndrome with much higher morbidity and mortality[50].

With the increasing practice of living-donor liver transplantation and the enlargement of the resectable limit, the small-for-size syndrome has emerged as an important clinical problem[51]. Although the pathogenetic causes of the small-for-size syndrome are still debated, it is assumed that the syndrome is primarily linked to portal hyperperfusion with high intravascular shear stress[52-54]. As a consequence of portal venous hyperperfusion, however, HABR may lead to hepatic arterial hypoperfusion of reduced-size livers (Figure 1B). In line with this, Smyrniotis *et al*[55] have shown in a porcine study that portal flow to split grafts with a graft-to-recipient liver volume ratio of 2:3 and 1:3 showed an inverse relationship to graft size, for example, the smaller the graft, the higher portal blood flow. By contrast, arterial flow decreased proportionately to graft size. In addition, HABR, which is present in all split-liver transplanted pigs, has been found to be increase as the graft-to-recipient liver volume ratio decreases[55].

A comparable hemodynamic pattern of hepatic blood flow has been observed in living related liver transplantation, in which size disparity between graft and native liver is the rule and almost universal[56]. In patients with living right lobe living donor transplantation, the grafts are subjected to impressive, more than double, increases of portal blood flow (Figure 1A and B). In the absence of active regulation, arterial flow might be expected to double as well. On the contrary, striking decreases in arterial flow have been seen in right lobe grafts[57], which represent the HABR as a reflexive response to changes in portal blood flow, to maintain total blood flow within an acceptable physiological range[58]. Troisi *et al*[59] have reported mean recipient portal venous flow values in small liver grafts (graft-to-recipient body weight ratio < 0.8) at least three times higher than those recorded in donors. Simultaneously, hepatic artery flow is significantly reduced and results in a decreased contribution to the liver from 30% in donors to only 6% in the recipients. In a porcine small-for-size liver transplantation model, the portal-to-arterial flow ratio remains increased until 5 d after surgery, which is poorly tolerated by transplanted livers[60].

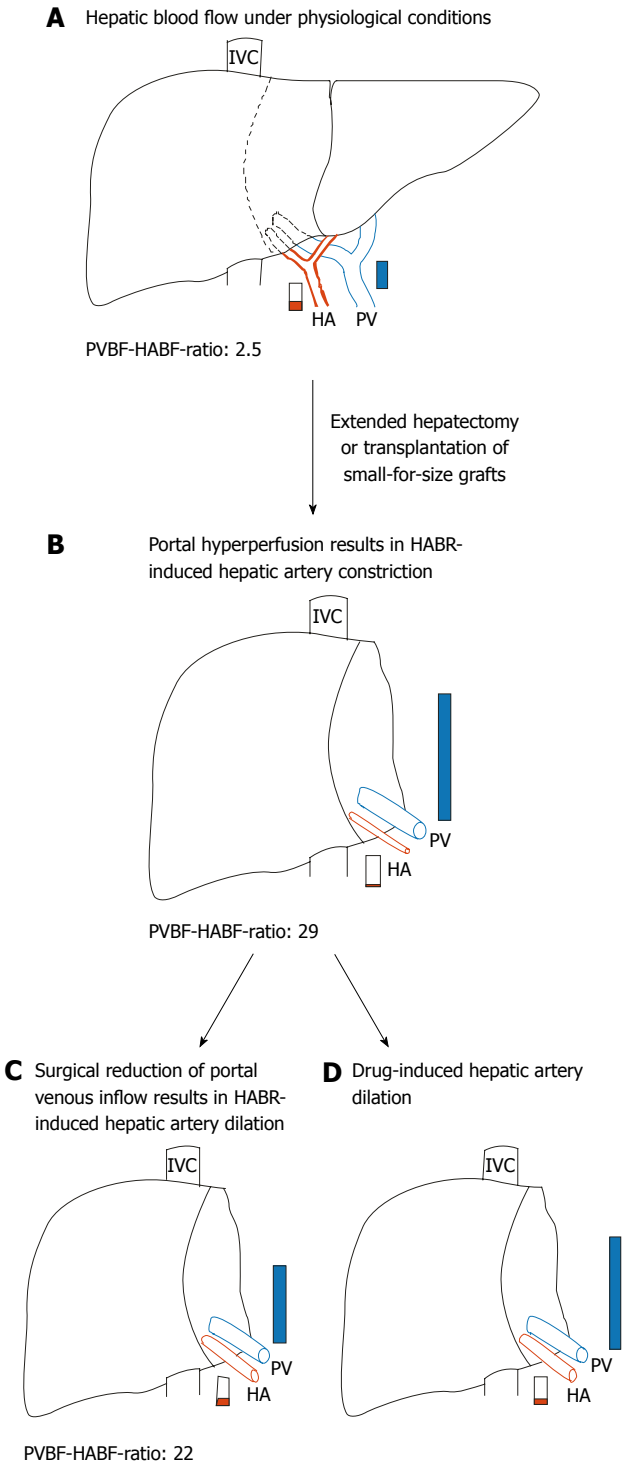

Figure 1 Hepatic hemodynamics in normal and reduced-size livers. A: Preoperative hepatic blood flow in a donor liver or before extended hepatectomy representing a normal portal vein blood flow-hepatic artery blood flow (PVBF/HABF) ratio of 2.5; B: As a consequence of portal hyperperfusion, hepatic arterial buffer response (HABR) leads to hepatic arterial hypoperfusion of reduced-size liver that is characterized by a dramatically increased PVBF/HABF ratio of 29; C: Surgical reduction of the portal venous inflow, for example, by splenectomy or hepatic artery ligation, leads to HABR-induced dilation of the hepatic artery and results in a reduced PVBF/HABF ratio of 22; D: Possible effects of pharmacological interventions to preserve hepatic artery supply. PVBF/HABF ratios are adopted from[59]. HA: Hepatic artery; IVC: Inferior vena cava; PV: Portal vein.

The HABR has been clearly demonstrated to be present also in patients after cadaveric liver transplantation[61]. Measurement of hepatic arterial and portal venous flow using ultrasound transit-time flow probes over the first 3 h after reperfusion has revealed a mean total liver blood flow of 2091 ± 932 mL/min, with a disproportionately high mean portal flow of 1808 ± 929 mL/min, which represents approximately 85% ± 10% of total liver blood flow. Correlation analysis has shown a positive correlation between cardiac output and portal venous flow, and a trend toward negative correlation between cardiac output and hepatic arterial flow[61]. In patients with a 50% reduced portal flow, Henderson and colleagues have reported a significant increase in hepatic artery flow, which indicates an intact HABR after cadaveric liver transplantation[61]. In line with this, Payen *et al*[62], by measuring hepatic arterial and portal venous blood flow during alternative clamping of both vessels every 12 h during 7 d in patients after orthotopic liver transplantation, have reported reciprocal increases of hepatic arterial flow only during selective clamping of the portal vein. By analysis of patients transplanted for liver cirrhosis, a high portal flow was present, together with an early increase of hepatic arterial resistance, which agrees with the HABR theory[63]. The presence of HABR in the transplanted liver is unequivocal[61,62,64], and because of liver denervation, it might be the only active mechanism that regulates liver arterial flow.

The consequences of inadequate hepatic arterial flow range from mild cholestasis to rapidly progressive graft failure[65,66]. In a porcine model of small-for-size syndrome, histological examinations of the grafts consistently confirm hepatic artery vasospasm and its consequences; namely, cholestasis, centrilobular necrosis and biliary ischemia[67]. In severe cases of small-for-size grafts, poor hepatic arterial flow and vasospasm lead to functional de-arterialization, ischemic cholangitis, and parenchymal infarcts[54,68]. Michalopoulos has concluded that the failure to regenerate is not different from the situation in which pHx is accompanied by ligation of the hepatic artery, which also results in failure to regenerate[68].

Prolonged CO_2 pneumoperitoneum in laparoscopic surgery reduces substantially the portal venous flow in humans, and the extent of the flow reduction is related to the level of intraperitoneal pressure[69,70]. HABR may serve for maintenance of total liver blood supply during laparoscopy-associated portal venous flow reduction. However, controversial data exist on the maintenance of HABR during high-pressure pneumoperitoneum. Yokoyama *et al*[71] have reported on activation of HABR in a rat model using fluorescent microspheres to measure splanchnic flow. Although portal venous flow decreased, the hepatic arterial flow was relatively preserved throughout all levels of intraperitoneal pressure studied. In contrast, Richter *et al*[72] have used ultrasonic flow probes in a rat model, and have shown reduced portal venous flow paralleled by a linear reduction of hepatic arterial flow during CO_2-pneumoperitoneum. HABR is also markedly impaired in cirrhotic rats undergoing CO_2 pneumoperitoneum[73]. Studies in large animals have revealed intact HABR with doubled hepatic arterial flow in neonatal lambs during abdominal distension[74], as well as loss of HABR with reduced hepatic arterial flow in pigs[75], or unchanged hepatic arterial flow in dogs upon CO_2 pneumoperitoneum[76]. In particular, head up body position leads to reduction in portal venous and arterial hepatic blood flow during elevated abdominal pressure[77]. Thus, head up position and intraperitoneal pressure elevation above 15 mmHg should be avoided during laparoscopic surgery to preserve hepatic blood flow[77,78].

HABR in inflammatory liver diseases

Owing to the scarcity of clinical studies on this subject, one must turn to experimental data, with reservations concerning their extrapolation to humans. In models of continuous intravenous infusion of *Escherichia coli* in rats, portal venous flow was reduced, and increased hepatic arterial flow resulted in unchanged total hepatic blood flow[79,80]. The increased hepatic arterial flow could be a result of an active HABR, although, in parallel, reports exist to demonstrate an increased hepatic artery flow without a reduction in portal venous flow during endotoxemia[81-83]. In a porcine model, it has been shown that endotoxin shock leads to time-dependent impairment of liver inflow beds, which results in increased portal venous back pressure and incremental resistance. The hepatic artery bed is dilated in the early phase of endotoxic shock but, over time, it is constricted[84]. There is ongoing discussion as to whether excessive production of nitric oxide is the cause of the endotoxin-induced alterations in hemodynamic homeostasis. While nitric oxide induces arterial hypotension and hepatic arterial vasodilation during endotoxic shock[85], ablation of the HABR has been shown to be independent of nitric oxide or an α-adrenergic-receptor agonist[84]. On the contrary, early administration of the nitric oxide donor sodium nitroprusside can reverse the negative effects on hepatic arterial flow induced by endotoxin[86,87]. Moreover, sodium nitroprusside partially reverses the detrimental effect of the nitric oxide synthase inhibitor L-NAME in experimental endotoxemia, which implies that the endotoxin-induced dysfunction of the HABR may be due to a selective inhibition of vascular endothelial function[87]. Furthermore, nitroprusside maintains mRNA levels of constitutive nitric oxide synthase in liver tissue that are decreased by endotoxin shock and tempers the burst in inducible nitric oxide synthase expression, thereby reestablishing the autoregulatory response of the hepatic artery following reduction of portal venous blood flow[86].

In turn, application of the vasopressin analog terlipressin during long-term hyperdynamic porcine endotoxemia significantly decreases portal venous flow, whereas hepatic arterial flow is markedly increased, which presumably reflects a restored HABR[88]. Furthermore, terlipressin attenuates the endotoxin-induced increase in exhaled nitric oxide[88], which points to the interaction between the vasopressin and the nitric oxide system in septic shock[89].

Almost no data exist on hepatic hemodynamics during conditions of acute or chronic viral hepatitis[90]. In addition, only a few studies have addressed hepatic hemodynamics under low-flow conditions, such as hemorrhagic shock. However, the data so far are consistent in that HABR is not abolished during sustained low abdominal blood flow[91-93]. In critically ill patients, mechanical ventilation has been found to decrease splanchnic perfusion. However, importantly, HABR is preserved and increased hepatic arterial blood flow compensates the decrease in portal blood flow under conditions of ventilation-associated positive end-expiratory pressure[94].

HABR in liver fibrosis and cirrhosis

The pathogenesis of liver fibrosis and cirrhosis is characterized by initial hepatocyte necrosis and inflammatory response, with subsequent activation of hepatic stellate cells and their transformation into myofibroblasts, which is responsible for excessive extracellular matrix synthesis and deposition. As a consequence, distinct alterations of the hepatic microvasculature, that is, rarefaction of sinusoids and structural changes of sinusoidal endothelia[95,96], result in deteriorated nutritive blood supply, increased total hepatic vascular resistance, and hence, portal hypertension and portosystemic collateralization[97]. Due to this increase in sinusoidal resistance, the capillarization of the hepatic microvasculature and the development of portocaval collaterals[98], portal venous blood flow progressively decreases in patients with cirrhosis[99,100]. An increase of hepatic arterial blood flow, that is, a decrease of hepatic arterial resistance, if it occurs, would indicate an activated HABR.

Studies in cirrhotic rats have underlined this hypothesis by demonstrating that, under baseline conditions, cirrhotic animals have higher hepatic arterial blood flow compared to controls[101]. Moreover, induction of HABR by a stepwise reduction of portal venous inflow causes a disproportionate increase in hepatic arterial flow in cirrhosis, which is further reflected by the significantly higher buffer capacity[101,102].

Although this concept has been well established, analyses in cirrhotic patients have produced conflicting results. Several studies have shown an increased hepatic arterial resistance in patients with cirrhosis. This is related to the degree of portal hypertension[103,104], portal resistance[104,105] and Child-Pugh score[104]. In contrast to these observations, a considerable body of evidence exists to indicate that, in cirrhosis, hepatic arterial vasodilatation occurs in response to reduced portal venous blood flow[106-109]. Accordingly, intraoperative measurements in patients with end-stage liver cirrhosis, who underwent living-donor liver transplantation, have revealed a continuously activated HABR under baseline conditions[109]. In these cirrhotic patients, the reduced portal venous blood flow is associated with an increased hepatic arterial blood flow (hepatic arterial to portal venous flow ratio = 0.88), which is in contrast to the relationship in healthy volunteers (hepatic arterial to portal venous flow ratio = 0.58)[109]. However, total clamping of the portal vein provokes a blunted response, as evaluated by the absolute and relative changes in hepatic arterial blood flow and by the buffer capacity[109]. In line with this, Iwao *et al*[110] have reported that the hepatic artery buffer index is significantly lower in cirrhotic than in control subjects. They have analyzed portal venous blood flow and hepatic artery pulsatility index as measures of hepatic artery resistance upon a 500-kcal mixed liquid meal consumption, which increases portal venous blood flow. They found an increase in hepatic artery resistance in all subjects, however, it was less pronounced in cirrhotic than control subjects[110,111]. Vice versa, the vasopressin-induced decrease of portal venous blood flow was met by a fall in hepatic arterial pulsatility index, which again was significantly lower in cirrhotic than control subjects[110]. In a large series of patients with advanced cirrhosis, who are undergoing transjugular intrahepatic portosystemic shunt (TIPS), Gülberg *et al*[108] have demonstrated that patients with hepatofugal blood flow show significantly lower resistance index before TIPS placement than patients with antegrade portal flow direction, and TIPS placement induces a significant decrease in the resistance index in patients with hepatopedal flow, but not in patients with hepatofugal flow. The fact that some degree of HABR is preserved even in patients with advanced cirrhosis and significant portal hypertension may further underline the biological need for this intrinsic mechanism. Although one might argue that the drop in resistance and thus increase in hepatic arterial flow may not fully compensate for the TIPS-induced reduction in portal blood flow, it has been shown that hepatic arterial vasodilatation provides substantial functional benefit in patients with cirrhosis, and that this effect does not depend directly on hepatic arterial microperfusion and is observed preferentially in patients with decompensated disease[107]. Thus, it is reasonable to state that the change in the ratio of portal venous to hepatic arterial blood flow in favor of the hepatic artery may sustain oxygen delivery and exert a protective effect on organ function and integrity. In line with this, portal vein occlusion does not cause deterioration in hepatic tissue pO_2 in the presence of HABR, although maximum buffer capacity of the hepatic artery was limited to 50%-60% in both cirrhosis and control animals, and total liver blood flow was found to be restored to only 71%-76% of baseline values[102].

MODIFICATIONS OF THE HABR

Surgical interventions for modification of the HABR

With the development of partial liver transplantation, either as living donation or as deceased donor split graft, much effort has been spent on improvement of surgical techniques. Full-right full-left splitting for two adult recipients is associated with risk of small-for-size syndrome, which manifests as a pattern of liver dysfunction associated with portal hypertension, diminished arterial flow,

delayed synthetic function, and prolonged cholestasis[112-115]. However, the present rates of splitting livers are too low in comparison with the calculated potential and it is to be expected that further improvement in the management of small-for-size grafts would bring splitting of the liver for two adult recipients within the reach of broad application[112]. Small-for-size syndrome is a clinical problem that is also observed after living donor liver transplantation and extended hepatectomy[52,116]. When the full portal vein flow has to transverse through a much reduced liver size, then the pressure building up in the portal vein effectively shuts down the flow through the hepatic arterioles and the liver becomes de-arterialized[68]. Arterial flow impairment appears as result of an active HABR, although in the past, reduced hepatic arterial flow has repeatedly been ascribed to the splenic artery steal syndrome[117-120]. This phenomenon describes the impaired hepatic artery flow by shifting of the main blood flow to the splenic or gastroduodenal artery in patients with hypersplenism. Quintini *et al*[121] have analyzed whole-organ liver recipients by Doppler ultrasonography, and have reported that hepatic artery vasoconstriction in response to portal hyperperfusion and exaggerated HABR produces a high resistive index with poor arterial perfusion. In all patients, splenic artery embolization reduces the resistance to distal hepatic artery flow by reducing flow in the splenic circulation and consequently in the portal vein. This has prompted the authors to revise the name of splenic artery steal syndrome to splenic artery syndrome, thereby underlining that the cause is portal hyperperfusion and not arterial siphon[121]. Most recently, a retrospective analysis of 650 orthotopic liver transplantations has revealed an incidence of 5.1% for splenic artery syndrome[122], which is well within the range of the estimated incidence of artery splenic syndrome of 3.1%-11.5% after orthotopic liver transplantation[117,118,123,124]. Prophylactic treatment with ligation of the splenic artery for all patients at risk for development of splenic artery syndrome is recommended and effectively prevents splenic artery syndrome[122]. In the case of postoperative diagnosis of splenic artery syndrome, coil embolization of the splenic artery can be recommended as the treatment of choice, with a low risk profile[122].

Clinical features of small-for-size syndrome are neither specific nor inevitable in low-weight livers, and many other factors than actual liver weight contribute to their occurrence. Among these, early elevation of portal venous pressure and persistent portal overperfusion most probably play a key role[48,49,125-128]. A reduction in the portal venous flow by means of splenic artery ligation, splenic artery embolization, or splenectomy has been shown to be efficient also in case of the small-for-size syndrome[48,59,127,129]. A case report by Lo *et al*[51] and prospective studies by Troisi *et al*[130] and Umeda *et al*[131] have shown that modulation of the recipient portal inflow by ligation or embolization of the splenic artery leads to an increase in recipient hepatic arterial inflow (Figure 1C), with improved liver function. The fact that splenic artery syndrome and small-for-size syndrome can be successfully

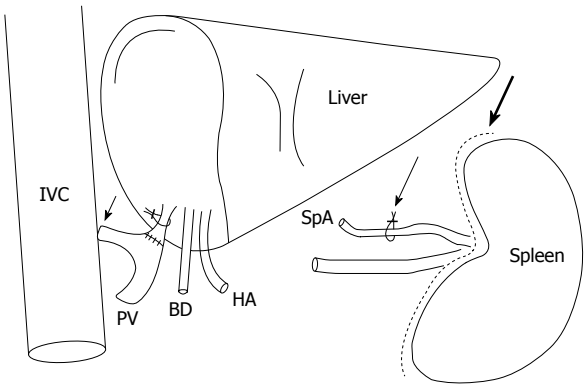

Figure 2 Surgical interventions for modulation of the hepatic inflow, showing the portocaval shunt (short arrow), ligation of the splenic artery (thin long arrow) and splenectomy (thick long arrow). BD: Bile duct; HA: Hepatic artery; IVC: Inferior vena cava; PV: Portal vein; SpA: Splenic artery.

treated by coil embolization of the splenic artery strongly underlines that both syndromes are pathophysiologically linked to the HABR. In line with this, detailed histopathological examination of sequential post-transplant biopsies and failed allografts with clinicopathological correlation has revealed that portal hyperperfusion, venous pathology, and the arterial buffer response make an important contribution to early and late clinical and histopathological manifestations of small-for-size syndrome[54]. In the most recent study of our group, we observed significantly increased survival of simultaneously splenectomized and hepatectomized rats compared to animals with 90% pHx alone[132]. It has been suggested that this effect is mainly caused by suppression of intrahepatic flow and less sinusoidal shear stress[49,55,133]. However, reduction of total hepatic inflow in simultaneously splenectomized and hepatectomized animals was marginal and not as pronounced as that required to improve survival by reduced shear stress. Instead, splenectomy before pHx caused a doubling of hepatic tissue pO_2 due to a HABR-induced rise of hepatic blood flow during extended pHx, which led to high tissue pO_2 values and reduced hypoxic stress. Supposedly, the increase of arterial inflow covers the oxygen demand and thereby improves organ regeneration and animal survival. Thus, improved arterial inflow rather than reduction of portal venous hyperperfusion is of great significance for the beneficial effect after inflow modulation in small-for-size livers[132].

Besides splenic artery ligation, established techniques such as portocaval or mesocaval shunts (Figure 2) may cause not only reduction of portal hyperperfusion, but also an increase of hepatic arterial inflow by reversion of the HABR (Figure 1C)[130,134-136].

In conclusion, the intraoperative measurement of both hepatic blood flows is important to predict the risk of small-for-size syndrome. The better ability to regulate finely the hepatic inflows would be useful in the treatment of liver dysfunction in settings of small-for-size transplantation, as well as extended hepatectomy, and necessitates further studies.

Pharmacological modifications of the HABR

The responsiveness of the hepatic artery to changes in portal flow is undoubtedly a desirable homeostatic mechanism under most circumstances, that is, the increase of hepatic arterial blood flow in case of reduced portal venous inflow. In contrast, the opposite situation, in which the dramatic excess of portal flow due to a smaller-than-average organ causes hepatic arterial constriction and hypoperfusion, might harm the liver (Figure 1A and B). Both extended hepatectomy and split-liver transplantation by fashioning two transplantable grafts from one liver result in small-for-size livers[50,116,137]. The regenerating liver requires an enormous amount of oxygen for its increased metabolic load and for DNA synthesis[138,139]. Suboptimal arterial inflow may be poorly tolerated in the reduced-size liver and increase the risk of organ dysfunction[60,68,138]. Possible pharmacological interventions could aim to enhance the hepatic arterial supply (Figure 1D). In line with this, in a porcine model of small-for-size syndrome, hepatic arterial infusion of adenosine significantly restored hepatic artery flow, reversed pathological changes in the graft, and finally improved survival[67]. In addition, an imbalance of vasorelaxing and vasoconstricting mediators is considered to be an important pathogenetic feature in reduced-size livers[140]. Maintenance of endothelin-1/nitric oxide balance by blocking endothelin A receptor reduces small-for-size injury by protecting the liver microcirculation and reducing hepatocellular damage[140]. Vice versa, substitution of nitric oxide has been shown to counteract the decline in hepatic arterial inflow in rats with 85% hepatectomy and cause a significantly greater increase in cell proliferation, with improvement of liver function[141].

Several programs in Japan have started clinical trials to reduce injury in small-for-size grafts by direct infusion of drugs such as prostaglandin E_1 and proteolytic enzyme inhibitors into the portal flow[126,142]. However, further experimental studies, including intraoperative measurement of both hepatic blood inflows, are warranted to clarify the precise strategies of pharmacological interventions and to select the appropriate drugs.

CONCLUSION

The crucial importance of the HABR as a regulatory mechanism to maintain adequate liver function and metabolic homeostasis has been recognized. Now, establishment of measures to modulate altered hemodynamics in small-for-size livers, as well as in cirrhotic and critically ill patients, warrants increased attention. Every effort for a timely diagnosis of altered or impaired HABR should be made in order to treat potential ensuing problems. Pharmacological and surgical interventions, which may be applied most easily, have to be proven in larger randomized clinical trials in order to improve the outcome of patients with liver disease with altered hepatic hemodynamics.

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TOPIC HIGHLIGHT

Rene Schmidt, MD, DESA, *Series Editor*

Molecular mechanisms of liver preconditioning

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Abstract

Ischemia/reperfusion (I/R) injury still represents an important cause of morbidity following hepatic surgery and limits the use of marginal livers in hepatic transplantation. Transient blood flow interruption followed by reperfusion protects tissues against damage induced by subsequent I/R. This process known as ischemic preconditioning (IP) depends upon intrinsic cytoprotective systems whose activation can inhibit the progression of irreversible tissue damage. Compared to other organs, liver IP has additional features as it reduces inflammation and promotes hepatic regeneration. Our present understanding of the molecular mechanisms involved in liver IP is still largely incomplete. Experimental studies have shown that the protective effects of liver IP are triggered by the release of adenosine and nitric oxide and the subsequent activation of signal networks involving protein kinases such as phosphatidylinositol 3-kinase, protein kinase C δ/ε and p38 MAP kinase, and transcription factors such as signal transducer and activator of transcription 3, nuclear factor-κB and hypoxia-inducible factor 1. This article offers an overview of the molecular events underlying the preconditioning effects in the liver and points to the possibility of developing pharmacological approaches aimed at activating the intrinsic protective systems in patients undergoing liver surgery.

Key words: Apoptosis; Hepatocyte; Hypoxia; Ischemia/reperfusion; Liver surgery; Necrosis; Pharmacological preconditioning; Preconditioning; Survival pathways

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INTRODUCTION

The understanding of the proteomic features associated with cell response to stresses is one of the present-day challenges in medical science. This knowledge is increasingly necessary to identify new molecular targets for therapeutic interventions. A turning-point on this matter has been the discovery that tissues already possess a number of inducible systems able to make them more resistant to a wide array of injuries. One of these adaptive responses is represented by the capacity of a non-lethal ischemia to modulate cell functions by increasing resistance to subsequent lethal ischemia/reperfusion[1,2]. Since its first description in the myocardium[1], this phenomenon, termed "ischemic preconditioning" (IP), has been the subject of

rising interest in the scientific and medical communities. The effects of IP can be differentiated into early effects and late effects. The former, immediately follows the transient ischemia and involves the direct modulation of specific cell functions, while late effects are evident within 12-24 h from the transient ischemia and require the simultaneous activation of multiple stress-responsive genes associated with the synthesis of several proteins[2,3].

ISCHEMIA-REPERFUSION INJURY OF THE LIVER

Hepatic ischemia/reperfusion (I/R) injury occurs as a consequence of trauma and hemorrhagic shock as well as temporary clamping of the hepato-duodenal ligament during liver resection (Pringle manoeuvre).

I/R is the main factor responsible for primary graft non-function or malfunction following liver transplantation[3,4]. Even moderate reperfusion damage, which does not severely affect the graft, can impair long-term hepatic recovery and enhance patient susceptibility to infections and multiple organ failure[3,4]. The shortage of organs for liver transplantation, forces consideration of cadaveric and steatotic grafts (marginal grafts) which have a higher susceptibility to I/R injury[4]. Living donor liver transplantation (LDTL) is a promising alternative approach aimed at increasing the number of donor livers[5]. A major concern over the application of LDTL in adults is graft size disparity which is responsible for the appearance of the life threatening effects of the "small for size syndrome"[6]. "Small for size syndrome" can occur even when the critical mass for safe LDTL (40% of standard liver volume) is transplanted and this effect is related to the impaired regeneration of the reduced liver mass[7] induced by I/R[7,8].

LIVER ISCHEMIC PRECONDITIONING

Beside the heart, IP effects have been demonstrated in many other tissues[2,3]. Studies performed in rats and mice, showed that interruption of liver blood supply for 5-10 min followed by 10-15 min of reperfusion reduced hepatic injury during a subsequent extended period of ischemia followed by reperfusion[9-13]. These beneficial effects were particularly evident in fatty livers in which preconditioning almost halved transaminase release and histological evidence of necrosis[11]. The application of preconditioning protocols to rodent liver transplantations showed that IP applied before cold preservation, decreased transaminase release and sinusoidal endothelial cell killing in the graft, improving rat survival[12,13].

A further feature of hepatic IP was the capacity to promote hepatocyte regeneration. Hepatocyte proliferation in rats subjected to 70% hepatectomy is significantly reduced by 45 min of hepatic ischemia. Such an effect was entirely reverted by pre-exposure to IP[14]. Consistently, preconditioning procedures significantly enhanced liver regeneration in the experimental model of reduced-size rat liver transplantation[15,16].

MOLECULAR SIGNALS OF HEPATIC ISCHEMIC PRECONDITIONING

Despite a significant number of studies on liver preconditioning, knowledge on the mechanisms responsible for the induction of the "preconditioned" phenotype is still incomplete. Studies from our and other laboratories have indicated that the process of preconditioning implies the production of complex proteomic modifications within liver cells which are now beginning to be characterized.

Adenosine, adenosine triphosphate and nitric oxide as molecular inducers of hepatic preconditioning

"*In vivo*" and "*in vitro*" studies have clearly established that the onset of IP is triggered by the production of adenosine and by the subsequent stimulation of adenosine A2a receptor (A2aR)[9,17-21]. In particular, Peralta *et al*[9,17] showed that adenosine treatment reproduced the protective action of IP and that IP was reverted by adenosine deaminase and by the adenosine A2 receptor antagonist, 3,7-dimethyl-1-propargylxanthine. Pretreatment of rats with the adenosine A2 receptor agonist, CGS21680, but not with the adenosine A1 receptor agonist, *N*-phenylisopropyl adenosine, enhanced tolerance against IR damage[18]. By using primary rat hepatocytes preconditioned with 10 min of hypoxia plus 10 min of re-oxygenation, we confirmed that the extracellular release of adenosine induced hepatocyte protection by autocrine stimulation of the A2aR[20,21] (Figure 1). Indeed, studies in extra-hepatic and hepatic tissues have clearly shown that transient oxygen deprivation triggers the release of several metabolites including adenosine triphosphate (ATP)[2]. In the extracellular space, ATP is rapidly metabolized to adenosine *via* CD39 and CD73 ecto-nucleotidases[22,23] present on the extracellular portion of cell plasma membranes. In particular, ectoapyrase (CD39) converts ATP to adenosine monophosphate (AMP), while ecto-5'-nucleotidase (CD73) further degrades it to adenosine[24]. Thus, CD73 represents the major extracellular pathway for adenosine generation. Consistently targeted gene deletion or pharmacologic inhibition of CD73 was demonstrated to abolish hepatic protection by IP[19].

Recent observations from our group also suggested that ATP itself could act as an additional trigger of liver preconditioning. We observed that the release of ATP from hepatocytes enhanced their tolerance to hypoxia independently from the generation of adenosine[24] (Figure 1). Such an effect was mimicked by treatment with the non-hydrolyzable ATP analogue adenosine-5'-O-(3-thiotriphosphate) (ATPγS) and involved the stimulation of the P2Y2 purinergic receptor[25].

Further evidence indicated that during IP, hepatic endothelial cells responded to adenosine stimulation by generating nitric oxide (NO) which contributed to the modulation of hepatocyte tolerance to I/R[3,17,26-28]. Indeed, the administration of NO donors promoted tolerance to I/R in the absence of adenosine, while NO synthase inhibitors reverted IP[17,26]. Similarly, the treatment of primary

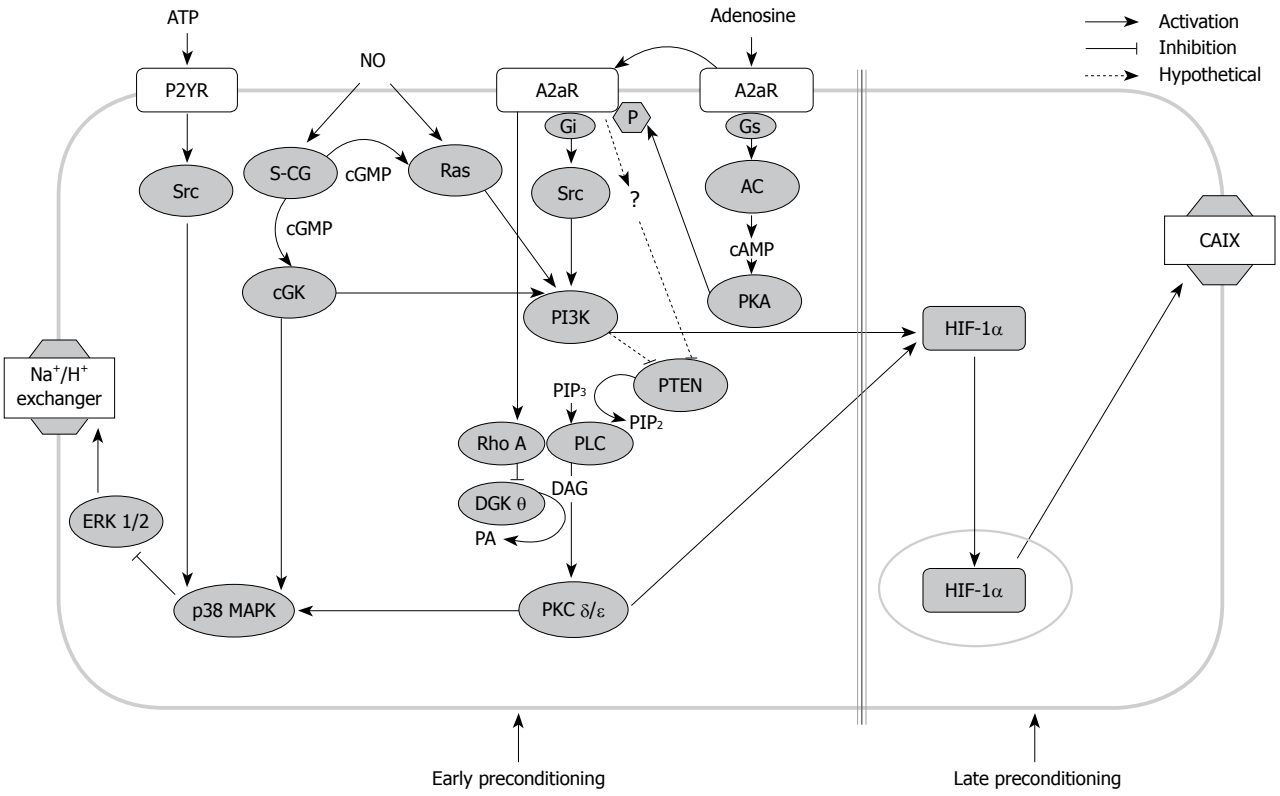

Figure 1 Signalling pathways involved in the development of ischemic preconditioning in rat hepatocytes. Adenosine triphosphate (ATP), adenosine and nitric oxide (NO) act as inductors of hepatocyte preconditioning by modulating a network of constitutive and newly synthesized signal mediators. Some of these mediators play a common central role in hepatocyte cytoprotection. p38 MAP kinase (p38 MAPK) is a mediator of the cytoprotective effects of all three preconditioning stimuli. Phosphatidylinositol 3-kinase (PI3K) mediates both adenosine and NO early resistance to hypoxia. PI3K together with protein kinase C (PKC) δ and ε, also induces hepatocyte late resistance to hypoxia contributing to the normoxic activation of hypoxia-inducible factor 1 (HIF-1). Diacylglycerol kinase theta (DGKθ) and the phosphatase tensin-homologues-deleted from chromosome 10 (PTEN) which metabolize diacyglycerol and phosphatidylinositol, respectively, are inhibited during preconditioning to sustain activation of the diacylglycerol (DAG)-dependent PKC δ and ε and the PI3K-dependent signals. See text and Refs[21,25,27,28,32,35,37,43]. P2YR: Purinergic P2Y receptors; A2aR: Adenosine 2A receptors; S-CG: Soluble guanylate cyclase; cGMP: Cyclic guanosine monophosphate; cGK: cGMP-dependent kinase; AC: Adenylate cyclase; cAMP: Cyclic adenosine monophosphate; PIP3: Phosphatidylinositol-3-phosphate; PKA: Protein kinase A; CAIX: Carbonic anhydrase IX; PLC: Phospholipase C; PA: Phosphatidic acid.

rat hepatocytes with the NO donor (Z)-1-{N-methyl-N-[6-(N-methyl-ammonio-hexyl) amino]} diazen-1-ium-1,2-diolate (NOC-9) reproduced hepatocyte resistance to hypoxic damage induced by IP, ATP or A2aR stimulation[27,28], suggesting that NO could act as an independent mediator of hepatic preconditioning[26].

Signalling pathways involved in adenosine and ATP-induced hepatoprotection

Using preconditioned rat hepatocytes, we observed that A2aR stimulation activated a cascade of intracellular signals involving Gi protein, phospholipase C (PLC), the novel isoforms of protein kinase C (PKC) δ and ε and p38 MAP kinase (p38 MAPK)[21] (Figure 1). The effective contribution of p38 MAPK in liver IP signalling was confirmed *in vivo* in mice where increased p38 MAPK phosphorylation was associated with tolerance against reperfusion injury[29]. Moreover, p38 MAPK inhibitors abolished resistance to I/R injury both "*in vitro*" and "*in vivo*"[21,30].

A2aRs are known to be typically coupled to Gs proteins that through adenylate-cyclase (A-C) stimulate protein kinase A (PKA)[31]. However, in an early study we excluded the involvement of PKA in mediating IP, as PKA pharmacological activation was devoid of protective action[21]. Subsequent research clarified this discrepancy, as we observed that A2aRs were actually coupled with Gs proteins and PKA[32]. PKA, however, by phosphorylating A2aR, shifted A2aR coupling from Gs proteins to Gi proteins and this led to the recruitment of the PLC-PKC pathway[32] (Figure 1). Interestingly, PKA phosphorylated A2aR only in the presence of its ligand (adenosine) and this explained why direct PKA activation in the absence of adenosine lacked protective activity[21,32] (Figure 1). The same research also highlighted the critical role of phosphatidylinositol-3-kinase (PI3K) in hepatic IP[32]. PI3Ks are a family of intracellular signal transducers that generate phosphatidylinositol (3,4,5)-triphosphate (PIP3), a second messenger that plays a central role in the regulation of cell proliferation, survival and metabolism[33]. In preconditioned hepatocytes, PI3K was activated upon A2aR engagement through Gi protein and Src kinase stimulation[32]. PI3K was shown to contribute to IP by promoting the activation of PLC and of PKC δ and ε (Figure 1)[32]. It is well known, however, that downstream of PI3K, protein kinase B (PKB/AKT) is a key modulator of a variety of pro-survival and pro-regenerative signals[33]. Thus, the PI3K-PKB/AKT pathway likely

represents an important pathway in the development of liver IP. Interestingly, PKB/AKT activation in connection with the development of tolerance to I/R was evident in rat hepatocytes and mouse livers[32,34] undergoing IP, as well as in preconditioned human liver grafts immediately after transplantation[35].

At present, the intracellular signals involved in ATP-dependent preconditioning are less well characterized. We reported that ATP-mediated activation of P2Y receptors was coupled with the phosphorylation of Src tyrosine kinase and of p38 MAP kinase that, in turn, inhibited the activation of ERK 1/2 consequent to hypoxic stress[25] (Figure 1).

Constitutive mediators of nitric oxide-induced cytoprotection

The signalling pathways responsible for the cytoprotective action of NO were investigated in rat hepatocytes treated with the NO donor, NOC-9, and then exposed to hypoxia. NOC-9-induced protection involved two parallel pathways. In one pathway, NO stimulated Ras GTPase, and in the other, NO directly activated the soluble guanylate cyclase (sGC) that by producing cyclic guanosine monophosphate (cGMP), stimulated the cGMP-dependent kinase (cGK) that also contributed to Ras GTPase activation[27,28]. Both the Ras and the cGK pathways then converged on the activation of PI3K, while only the sGC-cGK pathway was responsible for activating p38 MAPK (Figure 1)[27,28].

Negative regulators of liver preconditioning

It is increasingly clear that the development of hepatic IP requires the activation of a complex network of signals comprising cell-surface receptors, redox signals and a diverse array of protein kinases including PKCδ and PKCε. In preconditioned hepatocytes, the membrane recruitment and activation of PKCδ and PKCε was fully dependent on their direct interaction with diacylglycerol, generated by adenosine-induced activation of PLC-γ and diacylglycerol analogues which fully mimicked the activation of the signals that induce IP[3,21]. However, it is now clear that the accumulation of cellular diacylglycerol also depends on the rate of its metabolism to phosphatidic acid by diacylglycerol kinases (DGKs)[36]. In this regard, we recently observed that following IP or A2aR activation, the onset of hepatocyte tolerance to hypoxia was associated with a decrease in DGK activity[37]. Moreover, stimulation of A2aR specifically inhibited DGK isoform θ by activating RhoA-GTPase[37]. The pharmacological inhibition of DGKs has consistently led to a diacylglycerol-dependent activation of PKC δ/ε and of p38 MAPK. Moreover, both genetic and pharmacological inhibition of DGK θ induced cell tolerance to hypoxia[37]. Altogether these results unveiled a novel mechanism in the onset of hepatocyte preconditioning and demonstrated that the down-regulation of antagonist enzymes such as DGK was essential to obtain the diacylglycerol accumulation required to trigger PKC-mediated survival signals.

Similarly, preliminary data indicated that in parallel with the activation of PI3K, A2aR stimulation reduced the intracellular levels of the dual protein/lipid phosphatase tensin-homologues-deleted from chromosome 10 (PTEN) that inhibits PI3K-mediated signals by degrading phosphatidylinositol (3,4,5)-triphosphate[33]. We observed that PTEN inhibitors mimicked the induction of preconditioning (Cescon *et al*[35] unpublished results), while PKB/AKT activation and the clinical efficacy of IP in preconditioned human liver were fully explicated only in the presence of significant PTEN down-regulation.

Altogether these results demonstrated the importance of the down-modulation of key inhibitory enzymes for full activation of preconditioning responses. Moreover, these observations indicated the possible use of inhibitors of DGKs or PTEN as pharmacological inducers of hepatic preconditioning.

Nuclear transcription factors in liver preconditioning

As previously mentioned, the late effects of IP require the transcription of different stress-responsive genes and protein synthesis[2,3]. Growing evidence indicates that these responses are achieved by the coordinated activation of several transcription factors.

Nuclear factor-κB: Nuclear factor-κB (NF-κB) is typically devoted to the regulation of genes involved in inflammatory response and cell survival[38]. In experimental models of liver I/R, IP modifies NF-κB activity in different ways[29,39]. In one study, IP decreased NF-κB activity 1 h after reperfusion[39], and in another study, IP activated NF-κB during the ischemic period[29]. These contrasting results could be due to predominant NF-κB modulation in non-parenchymal *vs* parenchymal cells or to a differential regulation of NF-κB in the different phases of liver preconditioning. In addition, the NF-κB decrease during reperfusion was strictly related to a reduction in inflammatory cytokine expression[39], indicating a down-regulation of pro-inflammatory responses in Kupffer/sinusoidal endothelial cells. Conversely, NF-κB activation during ischemia was associated with the hepatoprotective action of IP[29], suggesting that, in hepatocytes, NF-κB-dependent genes contributed to survival responses.

Signal transducer and activator of transcription: The signal transducer and activator of transcription (STAT) transcription factors are a group of proteins implicated in the control of cell proliferation and survival processes[40]. IP induced the activation of the interleukin (IL)-6/STAT3 axis in liver and this pathway was involved in both cytoprotection and hepatic regeneration. On the one hand, as a result of hepatic preconditioning, NF-κB was shown to stimulate the expression of IL-6 and STAT3 that, in turn controlled cyclin beta1 synthesis and cell cycle progression[29]. On the other hand, studies with IL-6 null mice showed that the cytoprotective effects of IP against I/R injury depended on IL-6 signalling and were associated with hepatic STAT3 activation[41].

Hypoxia-inducible factor 1: Hypoxia-inducible factor 1

(HIF-1) is the main regulator of tissue adaptation to oxygen deprivation[42]. Active HIF-1 is a heterodimer consisting of an inducible HIF-1α subunit and a constitutively expressed HIF-1β subunit. HIF-1α is extremely labile in normoxia, as it is continuously degraded in proteasomes following hydroxylation, catalyzed by the oxygen-dependent HIF-prolyl-4-hydroxylase and arginyl-hydroxylase[42]. The lowering of intracellular oxygen prevents HIF-1α hydroxylation allowing its nuclear translocation and binding to hypoxic response elements of a number of genes regulating erythropoiesis, angiogenesis, glucose transport, glycolysis and cell survival[42]. Using preconditioned hepatocytes, we showed that HIF-1 activation was associated with the induction of a long lasting tolerance to hypoxic injury[43]. Furthermore, Amador and co-workers reported an increase in HIF-1α in concomitance with a lowering of hepatocyte apoptosis in human transplanted livers exposed to IP[44]. HIF-1 activation by IP was not due to the transient hypoxia occurring during the induction of preconditioning, but required A2aR activation[43]. This implicated an oxygen-independent mechanism in the regulation of HIF-1. Indeed, several reports demonstrated that a number of non-hypoxic stimuli (i.e. growth factors, cytokines, hormones and endotoxins) can activate HIF-1 in an oxygen-independent manner[45]. This process implies a PI3K- and PKC-dependent increase in the translation of HIF-1α mRNA, a process that shifts the synthesis/degradation balance towards HIF-1α accumulation[46]. We found that in hepatocytes, adenosine-dependent HIF-1 activation required the stimulation of both PI3K and PKC pathways[43]. This indicated that preconditioning stimuli, acting through the same survival pathways, could contextually lead to the early and late phase of response against cell injury.

Changes in the pattern of protein expression following liver preconditioning

Information concerning the genes modulated in response to liver IP is still limited. In accordance with the role of NO production as a trigger of IP, increased nitric oxide synthase expression was detected in preconditioned rat liver[47]. Microarray analysis of preconditioned human liver confirmed a significant increase in the amount of inducible nitric oxide synthase and also showed an increase in the anti-apoptotic protein, Bcl-2[48]. These analyses also showed that IL-1 receptor antagonist (IL-1Ra) was the most over-expressed gene in human preconditioned livers[48], in accordance with the anti-inflammatory effects of IP. Parallel studies investigating the gene expression pattern in preconditioned rat hepatocytes showed changes in 43 genes including those of the anti-inflammatory IL-10 and the antioxidant enzyme superoxide dismutase 2 (SOD2)[49]. In another study, a marked increase in SOD as well other endogenous antioxidants such as catalase (CAT) and glutathione peroxidase was also observed[50].

As previously mentioned, HIF-1 controls the expression of a variety of genes implicated in erythropoiesis, angiogenesis, glucose transport, glycolysis and cell survival[42]. In this context, we observed that the A2aR-dependent activation of HIF-1 in hepatocytes was associated with the expression of carbonic anhydrase IX (CAIX)[43], a transmembrane enzyme that by catalyzing bicarbonate production was implicated in preventing hepatocyte death (see later).

MOLECULAR MECHANISMS OF CELL RESISTANCE TO INJURY FOLLOWING HEPATIC PRECONDITIONING

The hepatoprotective effects of liver preconditioning impact on a number of different mechanisms. These include several processes acting against ischemia-induced damage as well as against reperfusion injury[2,3,50].

Protection against ischemic damage

A decrease in hepatic energy state is the main cause of liver cell injury during ischemia. Oxygen deprivation causes loss of mitochondrial potential, ATP depletion and intracellular acidification which are turning points in the onset of irreversible liver cell injury[3,50,51]. In early research, we found that activation of the Na^+/H^+ exchanger in response to cellular acidosis combined with the inhibition of Na^+ extrusion by the Na^+/K^+ ATPase, resulted in Na^+ accumulation within hepatocytes[52] (Figure 2). Na^+ overload was a critical step in hepatocyte damage during warm and cold hypoxia, as well as at the beginning of re-oxygenation, and its prevention markedly delayed the appearance of necrotic cell death[52-60]. Indeed, increased Na^+ caused an irreversible influx of Ca^{2+} by activating the Na^+/Ca^{2+} exchanger[56] and deranged cell volume regulatory mechanisms that ultimately led to osmotic hepatocyte lysis[53,58]. In rat hepatocytes, IP or treatment with A2aR agonists, ATP analogues or NO donors all protected against hypoxia-induced Na^+ overload and such protection was causally associated with increased cell survival[20,21,25,27,28,43]. Interestingly, the maintenance of Na^+ homeostasis was achieved both in the early phase of hepatocyte preconditioning[20,21,25,27,28], as well as in the late effects[43] (Figure 2). In the early phase of IP, inhibition of the Na^+/H^+ exchanger and activation of the vacuolar ATPase (V-ATPase) were mainly involved (Figure 2). Indeed, in hepatocytes treated with ATPγS, activation of the P2Y receptors/Src/p38MAPK axis inhibited ERK 1/2-mediated activation of the Na^+/H^+ exchanger responsible for Na^+ influx during hypoxia[25] (Figure 2). Adenosine- and NO-dependent maintenance of Na^+ homeostasis in preconditioned hepatocytes depended on p38 MAPK and PI3K signalling and involved the neutralization of intracellular pH achieved by the activation and translocation on plasma membrane of the V-ATPase (Figure 2). V-ATPase, acting as an alternative pH buffering system, extruded protons thus avoiding the activation of Na^+-dependent transporters[20,27,28]. The mechanism of Na^+ maintenance during the late phase of IP involved the HIF-1-mediated expression of CAIX in hepatocyte plasma membranes. The bicarbonate generated by CAIX was transferred to the cytosol through the Cl^-/HCO_3^- exchanger and neutralized intracellular pH avoiding Na^+ influx[42] (Figure 2). Beside these effects on

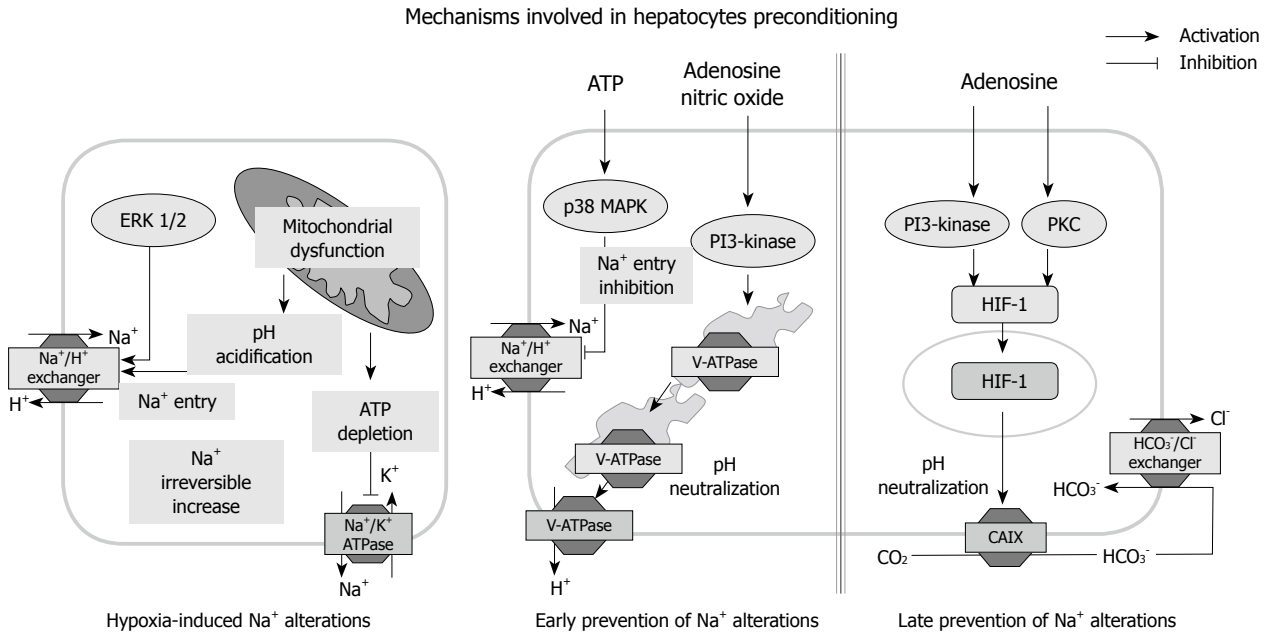

Figure 2 Na^+-dependent mechanisms involved in hepatocyte damage by hypoxia and their modulation by ischemic preconditioning. Hypoxia-induced adenosine triphosphate (ATP) depletion causes intracellular acidification, leading to inhibition of Na^+/K^+ ATPase and the activation of acid buffering systems (Na^+/H^+ exchanger). This leads to an increase in intracellular Na^+ that precipitates irreversible hepatocyte damage. ATP-dependent signalling through purinergic P2Y receptors prevents Na^+ accumulation by inhibiting the ERK 1/2-dependent activation of the Na^+/H^+ exchanger. Adenosine and nitric oxide (NO) activate the vacuolar proton ATPase that maintains intracellular pH avoiding activation of the Na^+/H^+ exchanger. Adenosine also induces the hypoxia-inducible factor 1 (HIF-1) target gene, carbonic anhydrase IX (CAIX), which converts CO_2 into bicarbonate, that once transported into the hepatocytes through the Cl^-/HCO_3^- exchanger, neutralizes the intracellular pH and prevents Na^+ accumulation. See text and Refs[20,21,25,27,28,43,52,53,58]. P38 MAPK: p38 MAP kinase; PI3-kinase: Phosphatidylinositol 3-kinase; PKC: Protein kinase C.

Na^+ homeostasis, during ischemia, IP also down-modulated hepatic energy metabolism by preserving the ATP and glycogen pools and limited lactate accumulation[61].

Protection against reperfusion damage

Mitochondria are a major target of the damaging effects of reperfusion[51]. Oxygen re-admission promotes free radical formation by uncoupled mitochondria with consequent mitochondrial oxidative damage and swelling[62]. IP protected mitochondria from oxidative reperfusion damage[63] and preserved mitochondrial redox-state[64], thus attenuating the impairment of ATP synthesis occurring at reperfusion. IP also improved hepatic intracellular oxygenation[65], preserved sinusoidal wall integrity and avoided liver microcirculatory failure induced by I/R[64]. Together these actions preserved aerobic ATP synthesis maintaining the hepatic energy status during re-oxygenation.

During reperfusion, preconditioned livers also showed a significant reduction in oxidative damage[66,67]. This effect could be ascribed to the increased content of antioxidant enzymes such as SOD, CAT and GSPx[49,50], as well as the reduced generation of reactive oxygen species by mitochondria and inflammatory cells. In the latter context, several studies have outlined the capacity of liver preconditioning to reduce inflammatory responses associated with reperfusion. IP decreased leukocyte adhesion to sinusoidal endothelial cells, lowering post-ischemic neutrophil infiltration[68,69]. IP also attenuated the production of pro-inflammatory cytokines/chemokines during reperfusion[10,68,69]. Finally, pharmacological stimulation of A2aR inhibited the activation of hepatic natural killer T lymphocytes, a process that was causally associated with the protective action of IP against hepatic reperfusion damage[70].

An important consequence of IP was the prevention of hepatocyte and sinusoidal endothelial cell apoptosis[3]. Such an effect can be ascribed to the amelioration of oxidative damage, to the reduced production of pro-apoptotic cytokines as well as to a direct interference with apoptotic mechanisms. Indeed, the increase in PKB/Akt observed in preconditioned hepatocytes[32] represents an important anti-apoptotic signal, since PKB/Akt blocks apoptosis by interfering with Bad, caspase-9 and cFLIP functions[71]. NF-κB might also be implicated in the regulation of hepatocyte response to pro-apoptotic stimuli and the increase in NF-κB nuclear binding observed as early as 30 min after liver IP[29] should be considered in this context. It cannot be excluded that NO-mediated signals might also contribute to the anti-apoptotic action of preconditioning by preventing loss of mitochondrial potential, cytochrome c release and caspase activation[72].

In conclusion, the combined effects of liver preconditioning on energy status, ion homeostasis, oxidative stress, pro-apoptotic responses and inflammation could explain the reduction in hepatocyte and sinusoidal endothelial cell death observed in preconditioned livers exposed to I/R[3,51].

Induction of hepatic regeneration

One of the key issues in the possible exploitation of preconditioning on LDTL is related to its effects on hepatocyte proliferation. The mechanisms involved in the pro-regenerative effects of liver preconditioning are beginning to be elucidated. Hepatocyte growth factor (HGF) and

transforming growth factor (TGF)-β are two cytokines that have opposite actions on liver regeneration, and promote and inhibit hepatocyte proliferation, respectively[73]. The capacity of IP to enhance liver regeneration after reduced-for-size transplantation was associated with increased HGF levels[15] and a lowering of TGF-β production[16]. These effects were causally related to a reduction in IL-1α and an increase in heat shock protein (HSP) 70 expression, respectively[15,16]. Furthermore, a recent study also associated the capacity of IP to attenuate injury in small-for-size liver grafts with the prevention of free radical production and mitochondrial dysfunction, through an increased expression of HSP90, a molecular chaperone that facilitates the mitochondrial import of Mn-SOD[74].

ISCHEMIC POST-CONDITIONING

The term ischemic post-conditioning refers to the capacity to prevent myocardial I/R injury by the application of brief cycles of ischemia during the reperfusion period after a sustained ischemic episode[75-77]. To date, the effects of post-conditioning in the liver have been reported in two studies. These studies showed that the application of brief ischemia in the early phase of reperfusion after rat liver transplantation, was associated with an amelioration of transaminase release and prevention of hepatocyte apoptosis[78-80]. These observations have new important clinical implications as these mechanisms may also act when hepatic damage has already started. In relation to the mechanisms involved in liver post-conditioning, preliminary results in our laboratory indicated that pharmacological post-conditioning with A2aR agonists induced PI3K activation and prevented post-ischemic damage in hepatocytes[81].

CLINICAL APPLICATIONS OF LIVER PRECONDITIONING

The clinical efficacy of hepatic preconditioning was clearly demonstrated in clinical trials performed in patients undergoing hemi-hepatectomy[4,82,83]. In these patients, IP obtained by 10 min of ischemia and 10 min of reperfusion before 30 min of inflow occlusion, significantly reduced transaminase release and ameliorated sinusoid endothelial cell apoptosis as compared to liver exposed to Pringle's manoeuvre only[82,83]. These effects were particularly evident in patients with mild or moderate steatosis, but were not observed in subjects older that 60 years[82]. Considering the possible impact that preconditioning may have in attenuating the effects of long-term graft exposure to cold and warm ischemia during liver transplantation procedures[2,4], the therapeutic use of IP in this setting should have important outcomes. The application of IP in human liver transplantation from deceased donors has, however, demonstrated conflicting results[44,84-88]. Indeed, some studies have shown the efficacy of IP in ameliorating transaminase release and in reducing primary graft malfunctions, whereas others have not observed significant differences[44,84-87]. In an attempt to gain some insight into the possible reasons for the failure of IP to protect liver grafts against reperfusion injury, we investigated the intracellular signals activated by IP in transplanted livers from heart-beating deceased donors. The data obtained indicated that IP stimulated PI3K-mediated signals in only half of the grafts and such variability correlated with the clinical effectiveness of IP. Our data also suggested that it was the failure of PTEN down-modulation that likely contributed to the lack of PI3K response to IP[35]. These observations indicated the necessity to explore alternative procedures to surgical IP to overcome the variability of human grafts in activating preconditioning responses. In this regard, the pharmacological induction of liver preconditioning likely represents a more reliable technique for stimulating the intrinsic systems of cytoprotection in humans.

PHARMACOLOGICAL INDUCTION OF HUMAN LIVER PRECONDITIONING

The clinical potential of pharmacological liver preconditioning is clearly suggested by animal studies, however, only two trials have so far addressed this aspect. In one study, Lang and co-workers reported that patients receiving volatile NO during orthotopic liver transplantation displayed an accelerated restoration of liver function as compared to the control group[89]. In the other report, Beck-Schimmer and co-workers showed that preconditioning with the halogenated anaesthetic, sevoflurane, in 64 patients undergoing liver surgery significantly ameliorated transaminase release and the incidence of severe post-operative complications[90]. These observations are consistent with increasing data regarding the efficacy of sevoflurane preconditioning in preventing myocardial ischemia/reperfusion injury[90]. Nonetheless, the availability of several liver specific NO donors and of a variety of effective adenosine A2A receptor agonists[91-93] offers the possibility of extending the number of studies aimed at directly evaluating new approaches to pharmacological liver preconditioning in humans.

CONCLUSION

In spite of a large number of studies on liver preconditioning, general knowledge on this phenomenon is far from complete. The available data give some insight into the signalling pathways responsible for both the early and late responses of IP, as well as some of the cellular modifications involved in the hepatoprotective effects of preconditioning. Additional extracellular inductors and constitutive or newly synthesized mediators are, however, likely to be involved. Little is known about the proteomic changes associated with inhibition of the inflammatory responses and the promotion of hepatic regeneration. Further research is thus needed to clarify these aspects. In particular, preclinical studies are necessary to identify a panel of the most suitable targets of liver preconditioning whose modulation by means of pharmacological or genetic therapies will allow effective activation of endogenous hepatoprotective systems in patients.

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TOPIC HIGHLIGHT

Rene Schmidt, MD, DESA, *Series Editor*

Heme oxygenase system in hepatic ischemia-reperfusion injury

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Abstract

Hepatic ischemia-reperfusion injury (IRI) limits access to transplantation. Heme oxygenase-1 (HO-1) is a powerful antioxidant enzyme which degrades free heme into biliverdin, free iron and carbon monoxide. HO-1 and its metabolites have the ability to modulate a wide variety of inflammatory disorders including hepatic IRI. Mechanisms of this protective effect include reduction of oxygen free radicals, alteration of macrophage and T cell phenotype. Further work is required to understand the physiological importance of the many actions of HO-1 identified experimentally, and to harness the protective effect of HO-1 for therapeutic potential.

Key words: Ischemia-reperfusion injury; Heme oxygenase; Transplantation; Ischemic pre-conditioning

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THE CLINICAL IMPACT OF HEPATIC ISCHEMIA-REPERFUSION INJURY

The rate of liver failure is increasing in the UK population[1]. Liver transplantation is an effective treatment for patients with end-stage disease, giving an average of 17-22 years of additional life[2,3]. Access to liver transplantation is limited by donor availability; several innovations, including split liver, living donor transplantation, non-heart beating donation (NHBD) and the expansion of the donor criteria, have been attempted to tackle this disparity[4].

Ischemia-reperfusion injury (IRI) causes a spectrum of early organ dysfunction after transplantation; the most severe form, termed primary non-function, may result in patient death. "Marginal organs", including those from older donors, those affected by steatosis and those from donors with long pre-donation intensive care unit stay, may be judged to pose an excessive risk of IRI and be discarded, placing additional pressure on the already scarce donor resource[5].

Due to shifting patterns of organ donation, there is a tendency towards the increased use of marginal organs. Year on year, the mean donor age is increasing, in part due

to improvements in road safety and declining numbers of traumatic deaths. Furthermore, NHBD (also termed donation after cardiac death) is becoming an increasingly important component of the donor resource[6]. Compared with the "gold standard" of heart beating donation (HBD), NHBD is associated with a decreased quantity of donated organs (2.1 organs per donor compared with 3.4 organs per HBD). Albeit in small studies, NHBD liver transplantation is also associated with a higher risk of IRI leading to elevated incidence of primary non-function[7].

Our group has previously estimated that negating the effects of IRI in HBD would lead to a 6% increase in the donor supply through recruitment of these marginal organs back into the donor pool[4]. In the current climate of rapidly increasing NHBD and increasing donor age, the imperative to better understand and avert hepatic ischemia is becoming ever stronger.

DEFINITION OF IRI

Interruption of blood flow to any tissue results in inadequate tissue oxygenation and an increase in cellular anaerobic pathways: if adequate oxygenation is not restored then disruption of cellular functions and cell death results. On reperfusion, despite restoration of adequate cellular oxygenation, there is further damage caused both by direct cytotoxicity from oxygen free radicals (OFR)[8] and by a secondary immunological assault upon the injured organ involving components of both the innate and adaptive immune system[9]. The sequence of injuries resulting from interruption then reinstatement of blood flow is termed IRI.

HEME OXYGENASE-1

Heme oxygenase (HO) is a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent microsomal enzyme, which catalyzes the breakdown of heme to biliverdin, iron and carbon monoxide (CO)[10] (Figure 1). Biliverdin is subsequently reduced to bilirubin by biliverdin reductase while free iron is sequestrated by ferritin. Of the three heme oxygenase isoforms (HO-1, HO-2 and HO-3), only HO-1 (also known as heat shock protein 32) is inducible[11]. HO-1 is a 32 kDa enzyme encoded by the *hmox1* gene. It has been found to be upregulated during states of oxidative and cellular stress and plays an important role in maintaining oxidative/antioxidant homeostasis[12].

Induction of HO-1 and its metabolites is protective in a large number of seemingly unrelated pathologies, including sepsis, malaria, endotoxic shock, IRI, organ transplant rejection, induction of tolerance, myocardial infarction, type 2 diabetes and obesity[13]. This spectrum of protection is attributed to multi-level mechanisms of cytoprotection and inflammatory modulation.

Polymorphism in the (GT)n microsatellite of the *HMOX1* promoter is thought to be responsible for the variations seen in the human response of HO-1 to stimuli[14]. This may account for the differences in the susceptibility of individuals to certain pathologies and in the apparent longevity associated with increased HO-1 expression[15,16].

HO-1 in hepatic IRI

In the context of IRI and transplantation, HO-1 induction or supplementation with its metabolites has been shown repeatedly to be protective in both the liver and other organs.

In early experiments, HO-1 was induced by heat shock in donor livers prior to 44 h of cold ischemia preceding liver isogeneic transplantation in rats, resulting in marked improvements in recipient survival[17]. HO-1 upregulation with adenoviral HO-1 or cobalt protoporphyrin (CoPP) resulted in improved portal venous flow on *ex-vivo* perfusion, while on transplantation into syngeneic hosts, recipient survival doubled, histological injury was ameliorated and influx of macrophages and T cells was reduced[18]; findings confirmed in similar experiments using transplantation[19,20] and hepatic warm ischemia models[21]. More recently, targeted deletion of *Bach-1*, which normally suppresses HO-1 transcription, led to HO-1 upregulation and protection from myocardial ischemia[22], although these experiments have yet to be repeated in models of hepatic ischemia.

In our own laboratory we have shown that HO-1 is upregulated in Kupffer cells during ischemic preconditioning (IPC)[23], and that targeted deletion of *hmox-1* resulted in aberrant Kupffer cell differentiation and susceptibility to ischemia-reperfusion insults[24]. Our work has suggested that Kupffer cells are a likely site of HO-1 action: recent work from Kupiec Weglinski's laboratory has demonstrated that adoptive transfer of HO-1 overexpressing bone marrow-derived macrophages was capable of protecting mice from hepatic ischemic insults[25]. Developing this theme, other experiments have shown HO-1 induction with CoPP to protect mice from hepatic ischemia arising from liver transplantation: Kupffer cells recovered from HO-1-induced animals produced less tumor necrosis factor α (TNFα) and interleukin (IL)-6 under stimulation in *ex vivo* culture[26].

HO-1 in hepatic IPC

IPC is a surgical maneuvre in which an organ is paradoxically protected by a brief period of controlled ischemia and reperfusion immediately prior to a longer index ischemic event, which by itself would normally lead to injury[27,28]. Several small randomized controlled trials have looked at the effectiveness of IPC in both liver transplantation and resection; subsequent Cochrane reviews conclude that further trials are still required to evaluate its role in hepatic and transplant surgery[29,30].

Kanoria *et al*[31] demonstrated in their rodent model that the application of a hindleg tourniquet (remote IPC) led to a protective phenotype from hepatic IRI. Ischemic post-conditioning has also been described, in which injury is abrogated by modified reperfusion. This emphasizes that tissue damage from ischemia-reperfusion continues following the end of the ischemic insult[32].

The mechanisms behind IPC are poorly understood. Numerous candidate molecules, including nitric oxide, adenosine, protein kinase C, tyrosine kinase and mitogen-activated-protein kinase, have been identified as potential mediators of protection[33]. Given that IPC can act re-

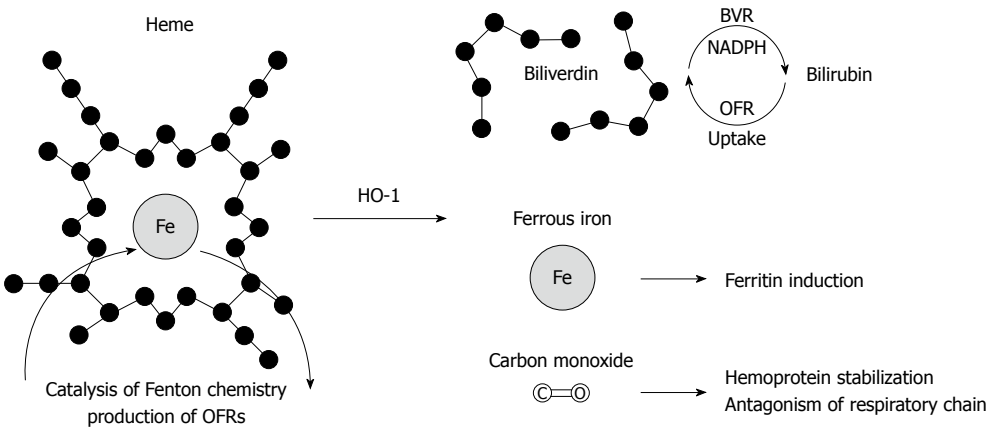

Figure 1 Putative mechanisms of heme oxygenase-1 antioxidant effect. HO-1: Heme oxygenase-1; BVR: Biliverdin; OFR: Oxygen free radicals; NADPH: Nicotinamide adenine dinucleotide phosphate.

motely, it may involve an immunomodulatory mechanism.

HO-1 is upregulated following IPC and may be responsible for the observed protection[23]. HO-1 is also upregulated in sites distant to the site of preconditioning. In a remote preconditioning model, renal ischemic insults led to cardiac HO-1 induction[34]. Likewise, HO-1 induction occurred in the liver following four 10 min episodes of femoral artery occlusion, and protection afforded by remote preconditioning was lost when HO-1 was inhibited with SnPP[35].

Given that HO-1 is upregulated in IPC and is known to be powerfully protective when upregulated, it is likely that HO-1 has a role in IPC together with other up- and downstream molecules.

Mechanisms of HO-1-mediated cytoprotection

As described above, it is now beyond doubt that HO-1 induction is protective in the context of hepatic ischemia and transplantation. HO-1's cytoprotective effects can be credited to a combination of removal of toxic metabolites (heme), and production of protective second messenger molecules in the forms of biliverdin (and subsequently bilirubin), CO, and free iron (which induces ferritin)[36]. HO-1 may also have other mechanisms of protection, independent of its enzyme activity[37].

Catabolysis of free heme

Heme consists of an iron atom contained within a porphyrin ring: heme moieties are usually contained within the "heme pockets" of hemoproteins. Although the most abundant hemoproteins are hemoglobin and myoglobin, heme moieties are contained within many other ubiquitous enzymes, for example iNOS and the mitochondrial electron transfer chain.

Under conditions of oxidative stress, hemoproteins may be oxidized, leading to the release of free heme which causes cellular injury by multiple mechanisms. Firstly, the central iron ion of the heme moiety catalyzes the production of free radicals by Fenton chemistry as follows: Step 1: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$; Step 2: $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\cdot + H^+$.

Heme-dependent free radical generation can cause direct cytotoxicity, including damage to the cytoskeleton, lipid bilayer, intermediary metabolic enzymes and DNA[38,39], while circulating heme can cause LDL oxidation leading to endothelial cell toxicity[40]. Heme sensitizes cells to pro-death signals including TNFα and Fas, an effect abrogated by treatment with antioxidants, implying dependence upon free radical production[36].

HO-1 reduces free heme concentrations by two mechanisms. Firstly, catabolysis of heme into metabolites biliverdin, iron and CO directly removes free heme. Secondly, binding of CO to hemoglobin, forming carboxyhemoglobin, prevents its oxidation to methemoglobin and ensuing release of free heme moieties[36].

Some authors have argued strongly that neutralization of free heme during cellular injury can account for a large proportion of the protection offered by HO-1. In experimental malaria, it was found that C57/Bl6J mice had reduced HO-1 induction compared with BALB/c animals, and consequently had higher levels of circulating free heme and greater disease severity. Administering exogenous heme to BALB/c animals caused worsening of their disease severity whereas conversely, CO treatment of C57Bl6J mice was protective[41]. In the same model, HO-1 protected animals from hepatic failure induced by overwhelming circulating heme[42].

Hepatic HO-1 expression is focused in Kupffer cells. This cell type is highly adapted to detect hemolysis: the hemoglobin-haptoglobin complex receptor CD163 is able to induce HO-1 in an IL-10-dependent manner[43,44].

Production of biliverdin

Biliverdin is produced by catalysis of the heme porphyrin ring and is almost immediately converted by biliverdin reductase to unconjugated bilirubin. This, in turn, is glucuronidated to render it water soluble. Bilirubin is a powerful antioxidant believed to be responsible for much of the antioxidant activity of serum[45]. At micromolar concentrations it is capable of scavenging large volumes of reactive oxygen species (ROS), protecting cells from high concentrations of peroxide, with enhanced efficacy in

hypoxic conditions, making this effect particularly relevant in the context of ischemia[46]. Bilirubin's extraordinary antioxidant capacity may arise from an active cycle in which biliverdin reductase undertakes NADPH-dependent reduction of biliverdin to bilirubin: in turn bilirubin is oxidized by free radicals back to biliverdin, before enzymatic reduction back to bilirubin. Although controversial[47], this cycling mechanism has been proposed as a highly adapted mechanism through which cells and whole organisms maintain redox status[45].

Bilirubin's effects may extend beyond its antioxidant action. It has been identified as an endogenous ligand of the aryl hydrocarbon receptor (AHR), present on many immune cell populations[48]. In some experiments AHR ligands have suppressed T cell proliferation through expansion of T regulatory (Treg) cells[49], although expansion of either Treg or their polar opposite, Th17 cells, has been shown depending upon which experimental AHR ligand is used[50].

Patients with persistent hyperbilirubinemia (Gilbert's syndrome) have been observed to have significantly lower incidence of atherosclerotic disease in several studies, an effect attributed to enhanced antioxidant potential of serum[51]. The effect of chronic hyperbilirubinemia upon T cell phenotype is unclear, although immunosuppression has been reported in patients with cholestatic jaundice[52].

Work using *ex vivo* liver perfusion models in which explanted livers were stored for 16[53] or 24 h[54] at 4℃ in University of Wisconsin solution, prior to mechanical perfusion, found that addition of low concentrations of bilirubin to the graft perfusate mimicked the protective effect of heme oxygenase induction. Graft function was improved in terms of portal venous flow and bile production, and hepatocellular injury was ameliorated in terms of histological injury scoring and transaminase release. Biliverdin has also been shown to have an immunomodulatory effect. In an MHC-mismatched cardiac allograft model, twice or three times daily injections of biliverdin for 2 wk increased graft survival, and led to complete allograft tolerance in 50% of animals[55].

To test whether bilirubin was protective in the clinical setting, our group hypothesized that hyperbilirubinemia would protect transplant recipients from IRI, in which case there would be an inverse correlation between preoperative bilirubin and post-operative transaminase measurements. In a small retrospective study, no relationship was observed, although given the heterogeneity of the study population an effect could not be ruled out[56].

Production of Fe^{2+}

Paradoxically for an antioxidant enzyme, one of HO-1's reaction products, free iron, is a powerful oxidant. Free iron catalyzes the generation of OFR by Fenton chemistry in a manner comparable to free heme as described above. However, it is more effectively neutralized than when contained within a heme ring, being actively exported from the cell[57] and chelated by iron-binding proteins, including ferritin. Ferritin sequesters Fe^{2+} by oxidation and placement of iron ions within a "core" in which they are unable to catalyze Fenton reactions[58]. HO-1 induction leads to increased ferritin expression[59], through a mechanism dependent upon the production of free iron[60,61].

Since other HO-1 metabolites had been shown to be capable of substituting for HO-1 induction in protecting animals from IRI, Berberat *et al*[62] hypothesized that ferritin overexpression could also confer protection. Using an *ex vivo* perfusion model of hepatic IRI, this group identified that transfection with adenoviral heavy chain ferritin conferred protection in terms of bile flow, portal blood flow, histological injury scoring and transaminase release, and improved survival of syngeneic liver recipients.

Ferritin induction has been observed in retinal[63] and cardiac IPC[64] in a manner dependent upon an iron signal[65]: although there are no published studies of ferritin expression in hepatic preconditioning, it would be reasonable to hypothesize that similar results would be obtained.

Although ferritin is capable of protecting cells and organs from oxidative stress, it is worth noting that its induction is unlikely to be the only mechanism of cellular protection by HO-1. Sheftel *et al*[61] compared heme and sodium arsenite (a non-heme inducer of HO-1) *in vitro*, and found sodium arsenite to confer protection through HO-1 induction without parallel ferritin induction.

Production of CO

CO is best known for its toxicity, causing death at atmospheric concentrations of 500-1000 ppm, however, at lower doses CO has been shown to have important cytoprotective and immunomodulatory functions: it is released by HO-1 during the catabolysis of heme and can substitute for the protective effect of HO-1.

CO cannot have a specific receptor since it binds only to transition metals and is not thought to be capable of direct interaction with amino acids. Therefore, its pharmacology as a signaling molecule is necessarily novel, and subject to ongoing debate. An important hypothesis is that CO effects must be mediated by interactions with proteins which contain transition metal cores, for example in heme rings contained within a range of enzymes including soluble guanylate cyclase (sGC), cytochromes, hemoglobin, myoglobin and nitric oxide synthase[66]. It has been suggested that CO protects from oxidative stress by preventing hemoprotein oxidation and subsequent release of heme rings[36], diminishing production of free radicals and subsequent apoptosis[67]. Others have suggested that the protection may be mediated by antagonism of respiratory chain enzymes, reducing cellular oxygen requirements[66], by inducing vasodilatation *via* sGC[68] or by opening calcium-sensitive ion channels[69].

Using an *ex-vivo* liver perfusion model, Amersi *et al*[70] demonstrated enhanced portal blood flow and bile production, and amelioration of hepatic IRI, in terms of histological injury score and transaminase release when perfusate was supplemented with CO at 300 ppm. Blockade of heme oxygenase using zinc protoporphyrin (ZnPP) did not obliterate the protective effect, indicating that CO

was capable of substituting for HO-1 function. Inhibitor studies have demonstrated that the CO effect was independent of iNOS and cGMP but dependent upon p38 MAPK.

In a cardiac transplantation model, no hearts stored at 4℃ for 24 h prior to implantation into syngeneic recipients functioned after implantation, whereas 5 out of 6 grafts survived when HO-1 was induced with CoPP, an effect lost with HO-1 inhibition. Administration of 400 ppm CO to the heart donor during cold storage resulted in survival equivalent to that achieved with HO-1 induction[71]. In xenotransplant models, HO-1 inhibition with tin protoporphyrin (SnPP) caused rejection, whereas this effect was overcome by recipient CO inhalation[72].

Although the finding that CO can be protective at low doses is scientifically exciting, the potential therapeutic use of inhaled CO can be limited by practical difficulties, both in control of dosage, and spillage of the gas into the environment around the patient. For this reason, Motterlini has developed transition metal carbonyl "CO releasing compounds" (CORMs). Of these (CORM-A1, CORM-2, CORM-3), the most commonly used has been CORM-3 [tricarbonylchloro(glycinato)ruthenium(II)], which releases CO when dissolved in saline but not water[73]. To control for the presence of the ruthenium compound, a CO depleted substance (iCORM3) can be prepared by dissolving CORM-3 in PBS prior to the experiment.

Using a coronary occlusion model in which mice were subjected to 30 min of ischemia followed by 24 h of reperfusion, investigators administered CORM-3 during the first hour of reperfusion, which halved the area of myocardial infarct compared to iCORM control[74]. In a further study, the same group compared the effects of CORM-3 with a late-phase IPC protocol. Animals received either IPC or intravenous CORM-3 infusion lasting 60 min, with appropriate controls. Animals then underwent 30 min coronary ischemic insults 24 to 120 h later. The reperfusion phase lasted for 24 h post-operatively before animals were killed and hearts examined for infarct size. Both IPC and CORM-3 infusion resulted in cardioprotection at between 24 and 72 h compared with appropriate control groups[75].

In the context of transplantation, the potential for using CO to enhance graft function has been explored in various transplantation models. In the kidney, several studies have successfully protected grafts using storage media supplemented with CORM-3/CORM-A1[76] or CORM-2[77] for extended periods of cold ischemia prior to isogenic transplantation. In a vascular allograft model, donor HO-1 induction or CORM treatment reduced subsequent CD8 influx and intimal hyperplasia lasting until animals were sacrificed at 6 wk[78].

In the liver, CO persufflation of University of Wisconsin (UW) storage solution improved subsequent graft function[79], a result replicated using CORM-3 supplemented UW[80,81] after cold ischemic times of 48 h. Use of CO treatment in experimental liver transplant recipients has also been explored. Tomiyama *et al*[82] transplanted livers from wild-type donors into E-GFP transgenic rat recipients who received inhaled CO prior to and for the 24 h post transplantation. Grafts within CO-treated recipients were found to have reduced numbers of infiltrating CD45+ host leucocytes, while purified donor CD68+ Kupffer cells produced less IL-6 and TNFα: primary cultured *ex vivo* Kupffer cells from CO-treated animals secreted less IL-6 and TNFα in response to lipopolysaccharide (LPS) stimulus.

CO clearly has powerful antioxidant and anti-inflammatory effects in a variety of systems. For this reason, despite the difficulties presented by administering it in its inhaled form, phase 2 clinical trials are underway in renal transplantation (www.clinicaltrials.gov identifier NCT00531856), chronic obstructive pulmonary disease (NCT00094406) and post-operative intestinal ileus (NCT01050712). Further development of CORMs should make CO therapies more convenient, thereby widening their potential clinical applications: on the basis of the preclinical data presented above such applications would be expected to include hepatic IRI and transplantation.

Other mechanisms of HO-1-mediated cytoprotection

The bulk of data exploring mechanisms of HO-1-induced cytoprotection supports the concept that it is its antioxidant function which protects cells and animals from injury. Two separate pieces of data suggest that other mechanisms should be considered. Firstly, Ponka's laboratory have demonstrated that the amounts of intracellular heme available for degradation may be insufficient to account for HO-1's powerful antioxidant effects[61]. Secondly, Dennery's laboratory has shown HO-1 translocation to the nucleus after cellular hypoxia or heme treatment, suggesting a role in transcriptional regulation[37]. Developing this work, this group has published research using a reporter system with catabolically inactive HO-1 which suggests HO-1 may have a forward-acting positive effect upon its own transcription[83], although as yet there has been no work demonstrating a protective effect of catabolically inactive HO-1.

HO-1 AND IMMUNOMODULATION IN IRI

It is likely that HO-1 protects organs from IRI by modulation of both direct cellular injury and both the innate and adaptive immune systems[84]. Deficiency of HO-1 in both humans and mice is associated with a pro-inflammatory phenotype[85,86].

According to Matzinger's "Danger Model" of the initiation of immune responses to injured organs, injured parenchymal cells release "Danger-Associated Molecular Patterns" (DAMPs) which stimulate antigen presenting cell (APC) activation through pattern recognition receptors[87,88]. Activated APCs subsequently activate and recruit immune effector cells through the secretion of pro-inflammatory mediators (cytokines, chemokines and adhesion molecules) and antigen presentation[9], resulting in

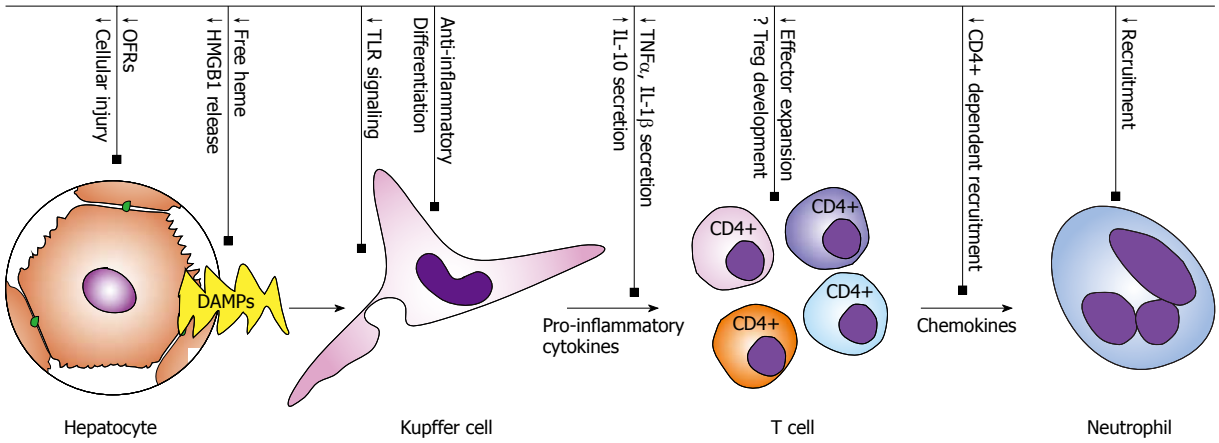

Figure 2 Heme oxygenase-1-mediated suppression of the inflammatory response in hepatic ischemia-reperfusion injury. HMGB1: High Mobility Group Box-1; TLR: Toll-like receptor; DAMPs: Danger-associated molecular patterns; TNFα: Tumor necrosis factor α; IL: Interleukin; OFR: Oxygen free radicals.

the activation and recruitment of T cells and neutrophils (Figure 2). This recruitment of leucocytes into the tissue is further facilitated by increased endothelial permeability and adhesion molecule expression resulting from endothelial activation.

Danger-associated molecular patterns

Hypoxic cellular injury can be regarded as the initiating event in a cascade of immunological activation. "Danger-Associated Molecular Patterns" or "alarmins" spilt by dying cells act on tissue resident immune populations *via* various molecular sensors including the toll-like receptor (TLR) family and purinergic receptors. A prototypic "alarmin", High Mobility Group Box-1 (HMGB1) is released passively from injured cells, a signal amplified by active secretion from surrounding viable immune and non-immune cells[89]. Several authors have published studies demonstrating that circulating HMGB1 levels rise after experimental hepatic ischemia and that anti-HMGB1 neutralizing antibodies are capable of lowering circulating TNFα and IL-6 levels, and ameliorating injury[90-92]. Human clinical studies have confirmed the synthesis of HMGB1 during early reperfusion after liver transplantation, an effect exaggerated in steatotic livers, and with levels correlating with injury severity as measured by peak ALT[93]. Recombinant HMGB1 has been found to worsen the severity of experimental hepatic ischemic injury, an effect neutralized by targeted deletion of its receptor TLR-4[92].

HO-1 is capable of modulating the HMGB1 alarmin system. In sepsis[94] and acute lung injury[95] models, macrophage LPS-mediated HMGB1 release was suppressed by HO-1 induction by CORMs resulting in improved survival. Oxidative stress can initiate macrophage HMGB1 synthesis which occurs in a dose response relationship with peroxide stress *in vitro*[96]. It is therefore unclear whether HO-1's modulation of HMGB1 secretion arises simply by reducing the extent of parenchymal cell injury or whether it is dependant upon another mechanism.

Free heme is another potential DAMP, being released from damaged cells and acting on TLRs[97]. Heme has also been shown to induce neutrophil chemotaxis using *in vitro* transmigration assays and after intraperitoneal injection *in vivo*[98]. By metabolising free heme, HO-1 induction would be expected to reduce this immune stimulus, further contributing to the possible mechanisms of protection from injury.

Intriguingly, HO-1 and CO also modulate TLR signaling upon which many DAMPs converge[99]. CO treatment of cultured RAW264.7 macrophages was found to reduce TLR4 signaling by reducing TLR4/Myd88 interactions and movement of TLR4 receptors to the cell surface[100].

It is likely that much of the immune modulation offered by HO-1 is owed to its antioxidant action which quietens the initial immune stimulus by reducing cellular injury and spillage of DAMPs.

Kupffer cells

Hepatic tissue resident macrophages (Kupffer cells) are the first immune cells to be activated by IRI, being acted upon by DAMPs released from surrounding parenchymal cells as well as being subject themselves to cellular hypoxia. Subsequently, they coordinate an appropriate influx of other immune cells by secretion of chemokines and cytokines. Kupffer cells have the ability to harm surrounding parenchymal cells by secretion of pro-inflammatory cytokines including TNFα, IL-6 and IL-1, which may be directly toxic[101]. On the other hand, Kupffer cells may protect the tissues by secretion of anti-inflammatory cytokines including IL-10. Furthermore, as the principle HO-1-expressing cells of the liver[24] they may secrete diffusible CO which may act in a paracrine manner upon surrounding cells to protect them from oxidative stress.

In our work, we have identified that HO-1 acts as a powerful switch on resting macrophage differentiation. We found HO-1-deficient Kupffer cells *in vivo* and bone marrow-derived monocytes (BMDMs) *in vitro* to differentiate down a Ly6c+ MARCO+ F4/80- pathway associated with macrophage inflammatory protein (MIP)-1 responsive pro-inflammatory monocytes[102]. HO-1 deficiency was associated with marked susceptibility to hepatic IRI measured in terms of ALT release and histological injury

score[24]. Parallel work elsewhere using an *in vitro* migration assay has demonstrated enhanced migration of *Hmox-1*$^{-/-}$ BMDMs towards MIP-1α[95]. In an experimental liver transplant model, inhaled CO conferred protection from injury and reduced secretion of pro-inflammatory cytokines from recovered CD68+ cells cultured *ex vivo*[82], reducing the subsequent influx of CD45+ leucocytes. *In vitro* and *in vivo,* low dose CO downregulates the production of macrophage pro-inflammatory cytokines [TNFα, IL1β and MIP-1β (CCL4)] and increases the expression of the anti-inflammatory cytokine IL10[103].

Data from injury models showing modulation of pro-inflammatory cytokine secretion by HO-1 induction is subject to the criticism that the changes observed are merely the downstream effects of parenchymal cellular protection, and suppression of DAMPs. However, data from our laboratory has shown HO-1 effects upon the resting state of macrophage differentiation implying that HO-1 may modulate immune responses themselves.

Adaptive immune system

There is growing evidence from a variety of animal IRI models (T cell-deficient nude rats, severe combined immunodeficiency mice, RAG 1$^{-/-}$ mice, CD4+/CD8+$^{-/-}$ mice and CD4+ depleted mice) that there is a significant decrease in biochemical and histopathological evidence of injury in the absence of T cells (reviewed by Linfert *et al*[9]). More specifically, Khandoga *et al*[104] demonstrated it was a CD4+ rather than CD8+ T cell-dependent phenomenon; depletion of CD4+ T cells leads to a significant reduction in the observed injury. This influx of CD4+ cells on reperfusion is rapid and may determine the mode of neutrophil activation and subsequently the extent of the observed tissue damage[105,106]. In a series of elegant adoptive transfer experiments with CD4+ cells in a renal model, Burne *et al*[107] found IRI to be an interferon-γ-dependent process; this may imply the importance of the T helper 1 lineage in the pathology of IRI.

HO-1 has a number of immunomodulatory effects on effector and Treg cells. HO-1 and CO appear to inhibit T cell proliferation and activation through the suppression of IL-2 secretion[108]. HO-1 induction has also been shown to induce the apoptosis of activated T helper cells through activation-induced cell death[109].

Treg cells suppress the activation of the immune system and inhibit the activation of autoreactive T cells[110]. In clinical studies, Treg/Th17 "imbalance" has been associated with acute coronary syndromes[111]. HO-1 is induced by FoxP3: inhibition of HO-1 function *in vivo* reduced the suppressive ability of naturally occurring Tregs[112]. Furthermore, studies from Fritz Bach's group have shown tolerogenesis to require HO-1[109]. Recently, however, these studies have been challenged by showing that *Hmox-1*$^{-/-}$ mice have normal numbers of Treg cells[113] and that HO-1 is not necessary or sufficient for T reg function[114]. These controversies may be resolved by work from the Agarwal group which showed that although *Hmox-1*$^{-/-}$ mice had normal (or elevated) numbers of circulating Treg cells, their suppressive function was dependant upon HO-1 expression by antigen-presenting cells[115].

In the context of hepatic ischemia, further work is needed firstly to establish which T cell populations are responsible for IRI and IPC, and secondly to resolve the controversy concerning the role of HO-1 in the expansion of different T cell subsets.

Neutrophils

Severe IRI is associated with significant influx of neutrophils[9]. Their recruitment from the vascular compartment into the liver is mediated by the expression of chemokines, cytokines and adhesion molecules[9]. Accumulation of activated neutrophils directly injures hepatocytes and the vascular epithelium through the release of proteases and the generation of ROS[116]. The initial recruitment of neutrophils appears to be a CD4+-dependent process[105]; continued neutrophil recruitment and activation may be dependent on the neutrophil production of IL-17A, which is important in a positive feedback mechanism[117]. As described above, heme is also capable of initiating neutrophil chemotaxis[98].

Although neutrophil influx is enhanced in *Hmox-1*$^{-/-}$ animals after ischemia-reperfusion insults[24], it is unclear whether this is due to a direct effect upon neutrophils or merely as a result of reduced stimulation due to upstream effects.

CONCLUSION

A wealth of data has now proven that HO-1, and its metabolites iron, bilirubin and CO, protect the liver from IRI. HO-1 appears to ameliorate ischemic injury through synergistic actions at many levels in the danger pathway. Powerful effects have been identified upon neutralization of OFR, and apparently at every step of the ensuing danger pathway. What remains is to understand the true physiological importance of each of these effects, and to attempt to separate apparent immunomodulation from suppression of upstream danger signals. Further understanding of the detailed pathophysiology of IRI and the mechanisms underlying IPC would also be invaluable in identifying the full range of potential therapeutic targets.

The protective effect of HO-1 upregulation in IRI was first discovered over a decade ago, and is proven beyond doubt in preclinical studies. Work to date has highlighted multiple potential therapeutic strategies ranging from CORM infusions to cell therapies with HO-1-transduced macrophages. In light of the severity of the many clinical problems caused by IRI including the liver donor shortage, there is now some urgency to translate this body of scientific knowledge into viable treatments which will benefit patients.

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TOPIC HIGHLIGHT

Rene Schmidt, MD, DESA, *Series Editor*

Role of nitric oxide in hepatic ischemia-reperfusion injury

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Abstract

Hepatic ischemia-reperfusion injury (IRI) occurs upon restoration of hepatic blood flow after a period of ischemia. Decreased endogenous nitric oxide (NO) production resulting in capillary luminal narrowing is central in the pathogenesis of IRI. Exogenous NO has emerged as a potential therapy for IRI based on its role in decreasing oxidative stress, cytokine release, leukocyte endothelial-adhesion and hepatic apoptosis. This review will highlight the influence of endogenous NO on hepatic IRI, role of inhaled NO in ameliorating IRI, modes of delivery, donor drugs and potential side effects of exogenous NO.

Key words: Nitric oxide; Liver; Ischemia-reperfusion injury; Drug delivery

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INTRODUCTION

Ischemia-reperfusion injury (IRI) is a series of multifaceted cellular events that takes place on the resumption of oxygen delivery after a period of hypoxia. This injury could be severe enough to lead to a significant morbidity and mortality.

The liver may be involved in IRI in procedures that are associated with sequential vascular impediment and restoration of blood flow; for example hepatic resections and orthotopic liver transplantation. During these procedures, unclamping of the vascular inflow to the liver after a temporary period of cross clamping results in major hepatocellular damage.

Nitric oxide (NO) has various protective effects on cells during IRI. NO has been demonstrated to inhibit oxidative stress, cytokine release, leukocyte endothelial adhesion and apoptosis[1]. On a cellular-signaling level, NO effects are mediated *via* redox-sensitive sites, and include: inhibition of protein kinase C, activation of tyrosine kinase, inactivation of nuclear factor (NF)-κB and activation of G proteins[2]. Previous studies have demonstrated that a reduction of NO during hepatic IRI, generally *via* a reduction in endothelial nitric oxide synthase activity, leads to liver injury[3]. Inhaled NO or NO donor drugs are novel treatments that have been used clinically to attenuate liver IRI[4]. This review will discuss the pathophysiology of liver involvement during IRI, and the clinical use of nitric oxide in ameliorating the impact of liver IRI.

BRIEF REVIEW OF THE PATHOPHYSIOLOGY OF IRI

The complex mechanisms of IRI have been revealed by advanced molecular biology[5] (Figure 1). During the isch-

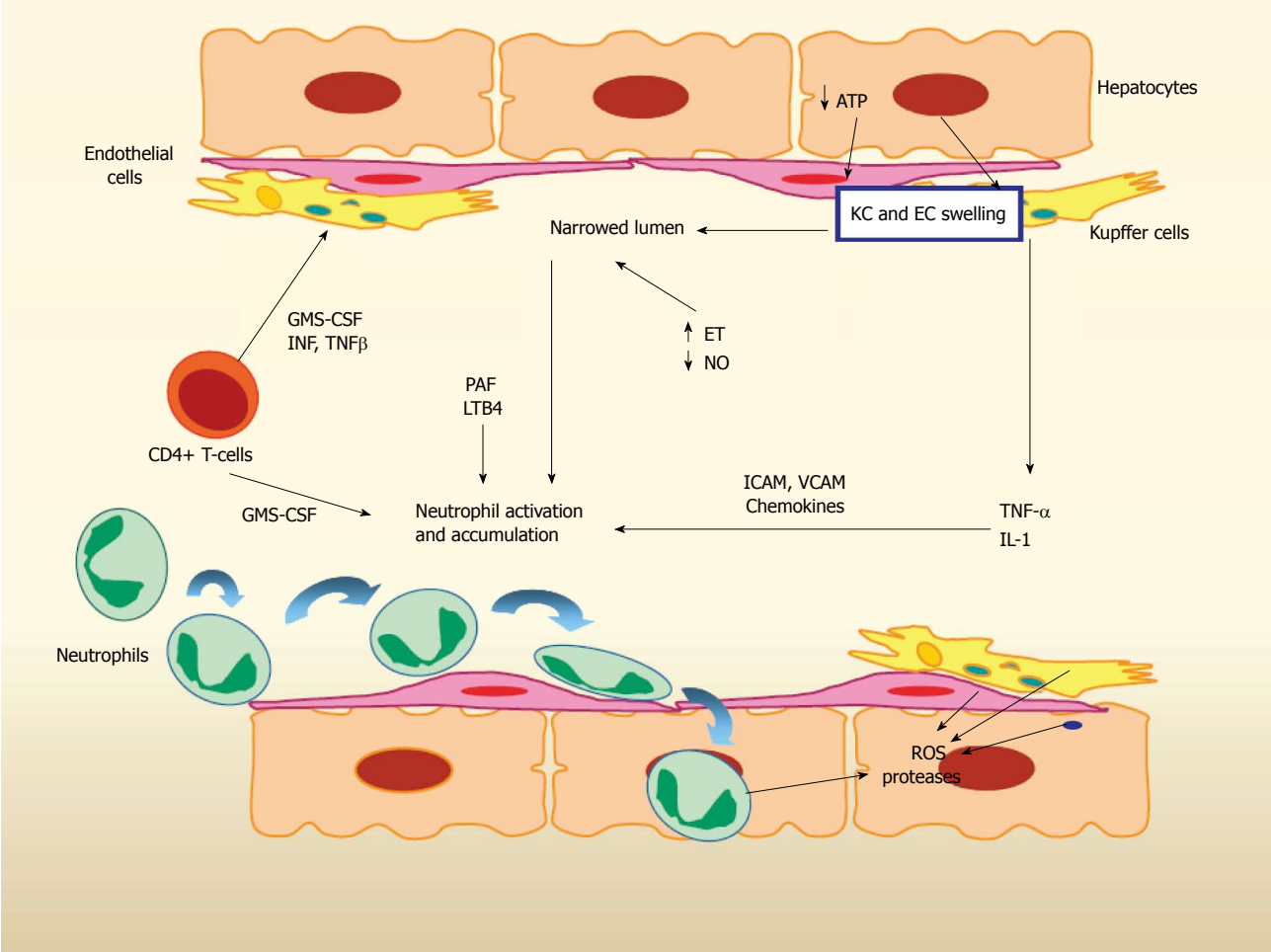

Figure 1 Multifaceted hepatic ischemia-reperfusion injury. Kupffer and endothelial cells produce cytokines and chemokines, recruiting neutrophils that further accentuate injury. EC: Endothelial cell; KC: Kupffer cell; ATP: Adenosine triphosphate; TNF: Tumor necrosis factor; IL: Interleukin; ICAM: Intercellular adhesion molecule; VCAM: Vascular adhesion molecule; PAF: Platelet activation factor; LTB4: Leukotriene B4; GMS-CSF: Granulocyte macrophage colony stimulating factor; INF: Interferon; ROS: Reactive oxygen species (Courtesy of Dr. Joan Rosello-Catafau, Barcelona, Spain).

emic phase, anaerobic metabolism ensues and produces an inadequate amount of high-energy phosphates which are fundamental to most cellular functions. Low levels of high-energy phosphates affect a myriad of cellular functions: homeostasis, signaling interactions, cellular proliferation and processing of the apoptotic death cycle. Adenosine triphosphate (ATP) depletion impairs sodium/potassium ATPase (Na^+/K^+-ATPase) function, resulting in an impairment of the efflux of sodium from the cell. Additionally, toxic metabolites, which are generated during ischemia, attract free water into ischemic cells and organelles leading to the formation of cellular edema[6]. If the ischemic insult lasts greater than 24 h, it is likely that ATP-synthase activity becomes irreversible after blood restoration, leading to cellular necrosis, apoptosis or neuroapoptosis[7]. Ischemia also causes an increased expression of adhesion molecules that leads to endothelial cell and neutrophil adhesion, resulting in vascular studding and occlusion[8]. Furthermore, disequilibrium between NO and endothelin (ET) induces vasoconstriction and subsequent microcirculatory failure even though blood circulation has been re-established[9]. Re-establishment of blood flow will serve to amplify inflammation with consequent injury that is highly variable but dependent on numerous variables including the extent of mediators produced (i.e. reactive oxygen species), the degree of endothelial and neutrophil adhesive responses and the degree of Kupffer cell activation.

PRINCIPAL PARTICIPANTS IN LIVER IRI

Sinusoidal endothelial cells

Injury to these cells is initiated during cold ischemia whereby Ca^{2+}-ATPase results in the accumulation of intracellular calcium[10]. Following this event, a series of actions occur making the endothelium more susceptible to platelet adhesion and reduced sinusoidal flow.

Kupffer cells

Kupffer cells are crucial in liver injury orchestration. Metabolic alterations of these cells occur during no-flow ischemia leading to the formation of reactive oxygen species during early reperfusion[11]. Additionally, at the onset of reperfusion, Kupffer cells undergo further activation by Toll-like receptor 4 signaling and/or by complement. Subsequently, Kupffer cells release pro-inflammatory cytokines such as TNF-α and interleukin-1 which them-

selves can perpetuate inflammatory injury by such means as leukocyte activation.

Hepatocytes

While major participants in the promotion of injury, during cold ischemia hepatocytes undergo intracellular bioenergetic perturbations that reduce ATP stores due to mitochondrial dysfunction and predispose these cells to injury during reperfusion[12].

Leukocytes and lymphocytes

As a result of IRI, cellular adhesion molecules (i.e. intercellular adhesion molecule-1 or ICAM-1, vascular adhesion molecule-1 or VCAM-1), selectins and integrins are activated and upregulated on the surface of endothelial cells, neutrophils and platelets. The activated neutrophils adhere to endothelial cells at the initial stages of reperfusion, and subsequently transmigrate across the endothelium where they continue to injury orchestration. The accumulation of activated neutrophils contributes to microcirculatory disturbances both locally and remotely. Activated neutrophils release reactive oxygen species, specifically superoxide radical (O_2^-•), proteases and various cytokines[13]. Monocytes and macrophages are also activated shortly following reperfusion[14]. Recent studies propose an important role for lymphocytes, especially $CD4^+$ T cells, in augmenting injury responses after IRI. However, lymphocytes may also play a protective role, but this is probably dependent on cell type and time course of injury[15].

Reactive oxygen species and reactive nitrogen species

During periods of ischemia, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated which can promote intracellular damage. Due to electron transport chain alterations, mitochondrial dysfunction ensues leading to reductions in ATP production and with subsequent loss of inner membrane stability resulting in mitochondrial swelling and rupture. With the reintroduction of oxygen during reperfusion, ROS are produced due to reactions of oxygen introduced during reperfusion with xanthine oxidase. ROS serve to stimulate other cell lines including Kupffer cells to produce proinflammatory cytokines[16]. The major ROS are hydroxyl radical (OH•) and hydrogen peroxide (H_2O_2). Reactions of ROS such as O_2^-• with NO yield products such as peroxynitrite ($ONOO^-$), a RNS which can be an extremely aggressive oxidant.

Cytokines

Cytokines play a vital role in IRI, both by inducing and sustaining the inflammatory response, and by modulating IRI severity. Tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) are the two cytokines most commonly implicated in liver IRI. TNF-α is a pleiotropic cytokine generated by various different cell types in response to inflammatory and immunomodulatory stimuli. TNF-α modulates leukocyte chemotaxis and activation, and induces ROS production in Kupffer cells[17]. Additionally, IL-1 is known to promote production of ROS, induce TNF-α synthesis by Kupffer cells and induce neutrophil recruitment[18].

Complement

The complement system also contributes significantly to IRI and is composed of approximately 30 soluble and membrane-bound proteins. This system can be stimulated in three pathways: (1) the antibody-dependent classical pathway; (2) the alternative pathway; or (3) the mannose-binding lectin pathway[19]. Complement, when activated, acts as a membrane-attacking complex that stimulates the production of proinflammatory cytokines and chemotactic agents. Furthermore, it can regulate adaptive immunity[20].

THE INFLUENCE OF ENDOGENOUS NO ON LIVER IRI

Damage to the liver due to IRI is a culmination of inflammatory cross talk with the principal participants mentioned previously. IRI is the main cause of liver injury in response to vascular clamping during hepatic procedures such as hepatectomy and liver transplantation. This insult on the liver results in disturbances of the sinusoidal microcirculation and the generation of a variety of mediators such as ROS, cytokines, activation of chemokines and other cell signaling molecules previously mentioned.

Hepatic IRI can cause severe hepatocellular injury that contributes to morbidity and mortality after liver surgery. As briefly mentioned previously, reductions of NO during liver IRI occur and are associated with increased liver injury[3]. This is now appreciated to be due to decreases in NO steady state production resulting from low concentrations of endothelium-derived nitric oxide synthase (eNOS). This event coupled with NO inactivation due to reactions with abundant ROS, such as O_2^-•, results in reduced NO bioavailability. The consequences of this reduced bioavailability include, but are not exclusive to, increased oxidative stress, increased apoptosis, increased leukocyte adhesion, increased microcirculatory tone, and perturbed mitochondrial function. Interestingly, restoration of NO to more "physiologic" concentrations serves to diminish the liver ischemic injury *via* countering of the adverse actions mentioned previously. Studies have demonstrated findings that are consistent with the premise that eNOS is crucial for minimizing injury during liver IRI. For example, liver injury was demonstrated to be less in wild type mice compared to eNOS knockouts ($eNOS^{-/-}$)[21] (Figure 2), in addition to the findings that agents given to increase eNOS expression or donate NO afford greater liver IRI protection[22,23]. It is also well established that the NO concentrations during various inflammatory states are significantly increased by increasing expression of inducible nitric oxide synthase (iNOS). However, the influence of iNOS and its true contribution in conferring liver protection (or not) deserves additional studies. In a rat model of liver IRI, iNOS expression was significantly increased correlating with increases in iNOS RNA at 1 and 5 h[24]. This is consistent with other studies measuring iNOS expression in conditions of liver IR. In

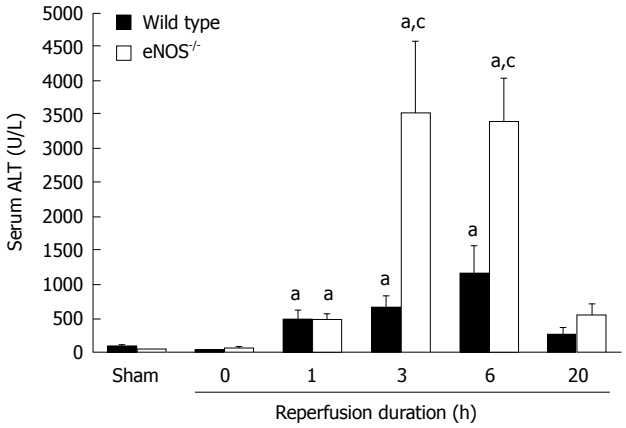

Figure 2 Increased liver injury as assessed by serum alanine aminotransferase in endothelium-derived nitric oxide synthase knockout mice compared with their wild type controls. $^{a}P < 0.05$ *vs* sham-operated controls. $^{c}P < 0.05$ *vs* time-matched wild type control. ALT: Alanine aminotransferase; eNOS: Endothelium-derived nitric oxide synthase (Courtesy of Dr. Ianes N. Hines, Chapel Hill, NC).

a porcine model of IRI, intraportal injection of the selective iNOS inhibitor, aminoguanidine, was demonstrated to decrease injury[25]. In an intriguing study, iNOS knockout mice (*iNOS*$^{-/-}$) exposed to warm liver IRI demonstrated a much greater magnitude of injury compared to wild type mice. Of notable interest was the finding that even though injury was greater in the iNOS knockout mice, little to no iNOS RNA was detectable in the wild type mice. It would appear that for now, the true influence of iNOS on liver injury during IR remains unclear.

A number of other endogenous NO-mediated mechanisms thought to confer protection have been published. For example, NO has been shown to inhibit caspase proteases *via S*-nitrosylation, thereby inhibiting apoptosis[26]. This appears to be somewhat concentration-dependent. Low physiological concentrations of NO may inhibit apoptosis. In contrast, higher concentrations may lead to the formation of toxic products such as $ONOO^{-}$ or other ROS which lead to cell necrosis and apoptosis[27]. Other published mechanisms of NO-mediated protection include inhibition of NF-κB[28], reversible inhibition of mitochondrial complex I, and decreased mitochondrial calcium accumulation[29]. As to be expected, controversy exists concerning "if" and "how" NO exerts cellular protection. For instance, in a study by Jaeschke *et al*[11], administration of a NO synthase inhibitor did not attenuate or accentuate liver injury during the initial reperfusion period. Inhibition of NO was observed not to influence neutrophil migration to the injured sites. While this contradicts a number of other studies, based on their findings, the authors concluded that NO availability was unlikely to be involved in the post-ischemic oxidant stress and reperfusion injury[30]. Nevertheless, the majority of published literature has demonstrated the beneficial effects of NO during liver IRI. These conflicting results might be explained by the fact that the mechanism of NO-mediated protection varies depending on cell type, quantities supplied, laboratory methods applied, timing and duration of NO exposure.

While iNOS was shown to be protective against hepatic IRI in some studies, it was shown to be deleterious in others. In a rat model of hepatic IRI, Takamatsu *et al*[31] observed increased hepatic expression of iNOS mRNA, ALT, and plasma iNOS at 3, 12, and 24 h after hepatic reperfusion. Concomitantly, there was evidence of histologic damage and nitrotyrosine formation in the liver sampled post-reperfusion. These changes were absent in the control group given the selective iNOS inhibitor, ONO-1714. The authors concluded that peroxynitrite may be involved in iNOS-mediated hepatic injury following IR[31].

In another model of hepatic IR in rats, Wang *et al*[32] observed an increase in iNOS protein and mRNA expression on the first day following hepatic reperfusion. Higher levels of iNOS correlated with evidence of increased hepatic injury in the form of elevated serum levels of ALT and AST. Administration of the non-selective nitric oxide synthase (NOS) inhibitor, L-NAME, significantly increased AST and ALT, while administration of the selective iNOS inhibitor, AE-ITU, significantly decreased AST and ALT levels, respectively[32]. The authors postulated that the deleterious effects of L-NAME were due to inhibition of eNOS, while the protective effects of AE-ITU were due to inhibition of injury-provoking iNOS. In a rat model of hepatic IR and small-for-size living-related liver transplantation, Jiang *et al*[33] observed increased iNOS mRNA and protein expression post-reperfusion from a warm ischemic insult with peak expression at 3 h post-reperfusion. This was accompanied by significant increases in concentrations of AST, ALT, malondialdehyde (MDA) and histologic evidence of damage compared to controls. The authors postulated that iNOS-induced hepatic damage was *via* significant production of ROS[33]. We summarize some key studies investigating endogenous NO and NOS in hepatic IRI in Table 1[3,21,25,31-37].

THE USE OF EXOGENOUS NO ADMINISTRATION IN ATTENUATING HEPATIC IRI

Inhaled nitric oxide

Inhaled NO was approved by the US Food and Drug Administration in December of 1999 for the treatment of persistent hypertension of the newborn. Over the last decade, the primary advantage of inhaled nitric oxide (iNO) was seen to be its ability to selectively decrease pulmonary vascular resistance with minimal effects on systemic blood pressure; however, there is currently much interest in exploring its other benefits, including its antioxidant properties and its cytoprotective abilities[4]. In many animal studies, iNO decreased infarct size and left ventricular dysfunction after IRI, increased coronary artery patency after thrombosis, increased blood flow in brain, kidney and peripheral vasculature, decreased leukocyte adhesion in bowel during ischemia-reperfusion, and decreased platelet aggregation[38]. Date *et al*[39] reported the use of iNO in 15 out of 32 patients who suffered from immediate severe allograft dysfunction, with iNO administered at 20 to 60 ppm. The mortality was significantly lower in the

Table 1 Effect of endogenous nitric oxide and nitric oxide synthase on liver ischemia-reperfusion injury

Species	Experimental methods	Ischemic time (min)	NO or NOS effects	Ref.
Pigs	Aminoguanidine, 5 min before ischemia	120	NO derived from iNOS, antioxidant	[25]
Dogs	FK 409, 30 min before ischemia and15 min before and 45 min after reperfusion	60	NO, improves hepatic microcirculation	[34]
Rats	L-arginine, 7 d before IRI	60	NO, antioxidant	[35]
Rats	L-NAME 60 min before ischemia	30	NO, antioxidant	[3]
Mouse	Gadolinium chloride 24 h before ischemia L-NAME methyl ester 15 min prior to ischemia	45	NO derived from eNOS, antioxidant, suppresses Kupffer cell function, regulated basal hepatic blood flow, but did not affect blood flow after reperfusion, attenuated neutrophils infiltration	[21]
Rats	L-arginine or Sodium nitroprusside or L-Name prior to ischemia	60	NO, improves peripheral liver blood flow after reperfusion, cytoprotective	[36]
Male rats	Arginine or L-NAME or 8-bromo guanosine 3′ 5′-cyclic monophosphate or rat atrial natriuretic peptide (ANP 1-28) 30 min before ischemia	45	NO, antioxidant, antiproinflammatory cytokines, improves microcirculation by the cGMP pathway, inhibits neutrophil infiltration and platelet aggregation	[37]
Male rats	IRI group: had partial clamping of portal vein and hepatic artery ONO-1714 group: as above plus ONO-1714 just prior to reperfusion and 6 h thereafter Control group: sham operation	90	iNOS expression peaked at 3 h and diminished at 24 h post reperfusion in IRI and ONO-1714 groups ONO-1714 significantly inhibited plasma nitrates at 24 h post reperfusion ONO-1714 significantly inhibited plasma ALT at 12 h post reperfusion, together with inhibiting histological damage and peroxynitrate expression in liver	[31]
Male rats	Microvessel clamping of portal vein and left hepatic artery L-NAME and AE-ITU given to each of 6 rats exposed to microvessel clamping (time unknown)	60	L-NAME worsened, elevated levels of ALT/AST in IRI groups AE-ITU mildly and significantly decreased levels of AST	[32]
Male rats	Portal vein, hepatic artery and bile ducts clamped by microvessel clamp followed by reperfusion	60	Significant elevation of AST/ALT, MDA/SOD in IRI and small-for-size liver transplantation groups	[33]

NO: Nitric oxide; NOS: Nitric oxide synthase; iNOS: Inducible nitric oxide synthase; eNOS: Endothelium-derived nitric oxide synthase; cGMP: Cyclic guanosine monophosphate; L-NAME: L-nitroarginine; ANP: Atrial natriuretic peptide; IRI: Ischemia-reperfusion injury; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AE-ITU: Aminoethyl-isothiourea; MDA: Malondialdehyde; SOD: Superoxide dismutase.

iNO group (7% and 24%, respectively). The gross benefits reported were that iNO improved oxygenation, decreased pulmonary artery pressure, shortened the period of postoperative mechanical ventilation, and reduced airway complications and mortality[39]. Likewise, a recent retrospective study also presented a picture of improvement of overall respiratory functions. The authors encouraged the administration of iNO for the prevention and treatment of early graft failure in lung transplant recipients[40]. Varadarajan *et al*[41] were the first group to study the relationship between NO metabolism and IRI in human liver transplantation[41]. From their study, they concluded that reduced bioavailability of eNOS contributed to IRI one hour after portal reperfusion. On the other hand, iNOS did not contribute to early IRI after human liver transplantation. Clinical and mechanistic reports on therapeutic use of iNO demonstrated action well beyond vascular relaxation, subsequently inactivated by oxy- or deoxyhemoglobin in the red blood cells. iNO has various positive effects on extrapulmonary systems. However, how iNO mediates these extrapulmonary effects remains unclear. Evidence supporting stable forms of iNO is probably strongest for *S*-nitrosothiols (SNOs) and nitrite[38]. In a prospective, blinded, placebo-controlled study, 80 ppm of iNO was administered to patients undergoing orthotropic liver transplantation[42]. Many advantages were reported in the iNO group, including reduced platelet transfusion, an improvement in the rate at which liver function was restored post-transplantation, and a decrease in the length of hospital stay. Most interesting was the finding of an approximate 75% reduction of hepatocellular apoptosis in patients treated with iNO[42] (Figure 3). Possible biochemical intermediates of iNO include plasma and red blood cell nitrate, nitrite, SNOs, C- or N-nitrosamines and red blood cell ferrous nitrosylhemoglobin. In this study, a detailed analysis indicated that the most likely candidate transducer of iNO in liver IRI was nitrite.

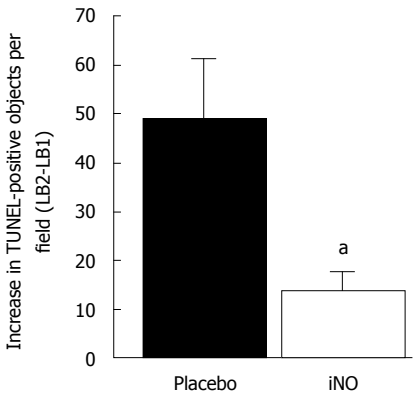

Figure 3 Decreased apoptosis indicated by TUNEL staining in patients treated with inducible nitric oxide compared to controls (Courtesy of John D. Lang, MD, Seattle, WA). $^{a}P < 0.05$. iNO: Inducible nitric oxide.

iNO delivery systems

An iNO delivery system should allow for constant and accurate measurements of NO and nitrogen dioxide (NO_2)

Table 2 Nitric oxide donors

Model	Drugs	Outcomes	Ref.
Canine liver IRI	FK-409	Promoted hepatic tissue blood flow, decreased serum endothelin-1, cytoprotection	[34]
Isolated hepatocytes	S-nitroso-N-acetylpenicillamine	Drug induced the expression of heat shock protein 70 mRNA and protein resulting in cytoprotection from TNFα	[2]
Murine liver IRI	Sodium nitroprusside	Promotes hepatic tissue blood flow after reperfusion-cytoprotection	[36]
Murine liver IRI	PEG-poly SNO-BSA, a sustained release of NO	Decreased neutrophil accumulation, prevented the excessive production of iNOS	[54]
Murine liver IRI	Macromolecule S-nitrosothiols	Prevented hepatocellular injury	[55]

NO: Nitric oxide; SNO: S-nitrosothiol; iNOS: Inducible nitric oxide synthase; IRI: Ischemia-reperfusion injury; TNF: Tumor necrosis factor.

concentration in inspired gas, as well as minimization of the contact time between oxygen and NO, in order to decrease the feasibility of producing high NO_2 concentrations. The measurement of iNO and NO_2 concentrations can be undertaken using chemiluminescence or electrochemical devices. There are some drawbacks of chemiluminescence devices such as cost, the need for a relatively high sample volume, noise and maintenance difficulties[43]. However, an electrochemical analyzer is relatively insensitive, and these measurements may be affected by pressure, humidity, temperature and the presence of other gases in the environment[44]. The delivery system should display the pressure of iNO in the cylinder and should have a backup power supply to avoid sudden discontinuation of iNO. Inhaled NO is usually supplied in nitrogen at various concentrations. The gas mixture concentration should be sampled downstream of the input port just proximal to the patient manifold. iNO also can be administered *via* nasal cannula, oxygen mask and oxygen hood[45]. Finally, the exhausted gas should be scavenged by passing it through carbon and filters, soda lime or activated charcoal[46].

POTENTIAL TOXICITIES DURING INHALATION

In the presence of high concentrations of O_2, NO oxidizes to nitrogen dioxide (NO_2). NO_2 reacts with the alveolar lining fluid to form nitric acid. NO dissolved in the alveolar lining fluid reacts with O_2^- yielding $OONO^-$, then decomposes into a hydroxyl anion[47]. Nitration of tyrosine residues of proteins is used as a marker of oxidative stress[48]. The rate at which NO is oxidized to NO_2 depends on the square of NO concentration and fractional concentration of oxygen to which it is exposed. The Occupational and Health Administration recommend 5 ppm exposure to NO per 8 h per 24-h-interval as the upper safe limit of human exposure[49]. In order to protect against NO_2 toxicity, iNO should be given with the least possible O_2 concentration. Inhaled NO and NO_2 concentrations should be monitored, exhaled gases should be scavenged, and a soda lime canister should be placed in the inspiratory limb of the breathing circuit.

Nitrite

The simple molecule nitrite had been thought to be just an index of NO production for decades[3]. Recently, a number of lines of evidence suggest that nitrite is a pro-mediator of NO homeostasis[50]. Administration of nitrite at near physiological concentrations (< 5 μg) leads to vasodilatation in animal and human studies[46]. Shiva *et al*[51] observed that nitrite was metabolized across the peripheral circulation. In addition, nitrite caused an increase in peripheral forearm blood flow when 80 ppm iNO was administered[51]. Under distinct conditions such as hypoxia and acidosis, nitrite can be reduced to NO by a number of deoxyhemeproteins (hemoglobin, myoglobin, neuroglobin and cytoglobin), enzymes (cytochrome P_{450} and xanthine oxidoreductase), and components of the mitochondrial electron transport chain[4]. Since nitrite can be converted back to NO during hypoxia, nitrite therefore is expected to be utilized during IRI. Furthermore, nitrite shows more potential benefits than NO in terms of safety and ease of administration. In other words, nitrite concentrations administered need only to be a small dose in order to increase plasma and tissue nitrite levels several folds. Routes of administration are oral, intravenous injection or infusion, intraperitoneal, *via* nebulizer or topical[52]. Nitrite has now been demonstrated to have cytoprotective effects in animal models of ischemia-reperfusion in organs. Duranski *et al*[52] evaluated the effects of nitrite therapy in *in vivo* murine models of hepatic and myocardial IRI, and showed that nitrite was associated with cytoprotective effects. In that setting, nitrite reduced cardiac infarct size by 67% and limited elevations of liver enzymes in a dose-dependent manner. These workers also demonstrated that nitrite was reduced to NO regardless of eNOS and heme oxygenase-1 enzyme activities[52]. The exact mechanisms as to how nitrite protects against this particular condition are being explored, but it appears that the benefit is mediated through the modulation of mitochondrial function by involving the posttranslational *S*-nitrosation of complex I to attenuate reperfusion oxygen radical generation and prevent cytochrome-C release[51].

NO donor drugs

Since nitric oxide is not considered to be an ideal gas for the treatment of IRI, NO donor drugs are now being explored as an alternative to the parent compound. Novel drugs have been developed and used for the delivery of NO in order to compensate for the very short half-

life of NO *in vivo*. However, there are only two types of NO donor drugs that are currently used clinically: organic nitrates and sodium nitroprusside. Organic nitrates are the most commonly used NO donor drug treatment for coronary artery disease and congestive heart failure because the drugs produce clear clinical responses through their vasodilatory effects. Preparations of drugs include slow release oral forms, ointments, transdermal patches, nebulizers and traditional intravenous forms. The main limitation of organic nitrates is the induction of drug tolerance with prolonged continuous use. NO release from nitroglycerin is likely *via* the enzyme, mitochondrial aldehyde dehydrogenase[53]. The mechanism of NO release from sodium nitroprusside, on the other hand, is more complex, as demonstrated by Yang *et al*[53] in a murine model of hepatic IRI. Sodium nitroprusside is thought to down-regulate the mRNA expression of several enzymes related to hepatic injury[54]. We summarize other novel NO donor drugs in Table 2[2,34,36,54,55].

CONCLUSION

Ischemia-reperfusion injury is a well-defined threat to the liver during periods of interruption and restoration of oxygen delivery, as occurs in certain procedures such as hepatic resections and orthotopic liver transplantations. Relative NO deficiency seems central in the pathogenesis of this injury. Replacing NO *per se* either by inhalation, nitrate anion or *via* donor drugs represents a novel means for ameliorating IRI. Further randomized controlled trials are needed to evaluate this therapy in patients undergoing operative procedures causing IRI.

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TOPIC HIGHLIGHT

Rene Schmidt, MD, DESA, *Series Editor*

Hepatoprotective actions of melatonin: Possible mediation by melatonin receptors

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Abstract

Melatonin, the hormone of darkness and messenger of the photoperiod, is also well known to exhibit strong direct and indirect antioxidant properties. Melatonin has previously been demonstrated to be a powerful organ protective substance in numerous models of injury; these beneficial effects have been attributed to the hormone's intense radical scavenging capacity. The present report reviews the hepatoprotective potential of the pineal hormone in various models of oxidative stress *in vivo*, and summarizes the extensive literature showing that melatonin may be a suitable experimental substance to reduce liver damage after sepsis, hemorrhagic shock, ischemia/reperfusion, and in numerous models of toxic liver injury. Melatonin's influence on hepatic antioxidant enzymes and other potentially relevant pathways, such as nitric oxide signaling, hepatic cytokine and heat shock protein expression, are evaluated. Based on recent literature demonstrating the functional relevance of melatonin receptor activation for hepatic organ protection, this article finally suggests that melatonin receptors could mediate the hepatoprotective actions of melatonin therapy.

INTRODUCTION

It has been suggested that the substance melatonin (5-methoxy-N-acetyltryptamine), discovered by Aaron Lerner in 1958, exists in almost every animal species, and possibly even in all plants[1,2]. Its physiological functions are said to be diverse; while melatonin may be involved in modifications of vasomotor tone[3,4] and thermoregulation[5], it is primarily known as the signal of darkness[6].

In vertebrates, melatonin is synthesized in the pineal gland and secreted during darkness as a hormonal message of the photoperiod[7]. The rhythm of melatonin synthesis is mainly driven by an oscillator which is situated in the hypothalamic suprachiasmatic nucleus (SCN)[8]. This oscillator is usually entrained to a 24-h rhythm by environmental lighting conditions, which are perceived in the retina by rods, cones and intrinsically photosensitive retinal ganglion cells[9].

Based on the photoperiodic information transduced from the retina *via* the SCN to the pineal gland, melatonin is secreted during darkness after *de-novo* synthesis from tryptophan[10]. This nocturnal melatonin signal is proportional to the length of the night, thus encoding not only

circadian, but also seasonal variations in the photoperiod[11]. In so-called photoperiodic animals, like the Siberian hamster, these seasonal variations in melatonin output may have a profound influence on the regulation of reproduction[12,13], prolactin secretion[14], as well as coat color[15]. The nocturnal secretion of melatonin is generally independent of an animal's active period: in both nocturnal and diurnal species, melatonin levels rise during darkness[6].

Melatonin synthesis is not exclusively located in the pineal gland, but has also been described in numerous peripheral organs, such as the retina[16], bone marrow[17], skin[18], Harderian gland[19], platelets[20], lymphocytes[21], testes[22], and in the gastrointestinal tract[23]. Data on messenger RNA expression of two key enzymes responsible for melatonin synthesis, arylalkyamine-N-acetyltransferase and hydoxyindole-O-methyltransferase, suggest that even more peripheral organs may be able to produce this hormone[24].

So far, the physiological significance of extrapineal sites of melatonin synthesis remains unclear. However, besides its relevance in the time-keeping system, melatonin has been demonstrated to be a powerful radical scavenger[25]; it is tempting to assume that extrapineal melatonin may serve as a tissue protective agent.

MELATONIN AS AN ANTIOXIDANT

Processes of acute inflammation, e.g. sepsis, hemorrhagic shock or ischemia/reperfusion, typically result in an imbalance of oxidative homeostasis with excess generation of reactive oxygen species (ROS) and a relative deficiency of endogenous antioxidants; this state is called oxidative stress. ROS include oxidants, such as peroxynitrite, and free radicals, such as hydroxyl radicals and superoxide; these substances are toxic and may induce lipid peroxidation (LPO), as well as protein, sugar and DNA degradation[26].

The powerful antioxidant capacity of melatonin is usually attributed to its potential to eliminate free radicals by the donation of electrons[27,28]. For example, melatonin may neutralize hydroxyl radicals by forming 3-hydroxymelatonin, which is excreted in the urine[29]. Furthermore, melatonin was demonstrated to interact with toxic reactants like peroxyl radicals[30], singlet oxygen species[31], and hydrogen peroxide[32]. Metabolites of melatonin, including the major hepatic metabolite 6-hydroxymelatonin, as well as N-acetyl-N-formyl-5-methoxykynuramine and N-acetyl-5-methoxykynuramine have been shown to detoxify radicals themselves[32-34]. This powerful pyramid scheme of radical scavenging has been named "the antioxidant cascade of melatonin"[1,34].

In addition to these direct interactions with ROS, melatonin may induce upregulation of the activity of antioxidants and antioxidant enzymes, such as superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPx) and glutathione reductase (GSR), in the environment of oxidative stress[35,36]. In addition, the pineal hormone may induce downregulation of pro-oxidant enzymes like nitric oxide synthase (NOS)[37,38] and lipoxygenases[39], thus reducing the formation of nitric oxide (NO), superoxide anions, and subsequently peroxynitrite anions.

Both the direct detoxification of radicals, as well as the modification of pro- and antioxidative enzyme activities are thought to be relevant for the pineal hormone to act as a protective substance, for example when administered in models of oxidative stress. This valuable effect appears to be independent of the type of injury and the species investigated. Exogenous melatonin may exhibit beneficial actions in a myriad of models of organ damage; this is especially true for the liver.

HEPATOPROTECTION BY MELATONIN ADMINISTRATION

With respect to its hepatoprotective effects, countless publications have demonstrated that exogenous melatonin may be used successfully to treat a great variety of different pathophysiological conditions[40-146]: Table 1 gives an overview of the hepatoprotective effects of exogenous melatonin administration, without the pretension of being complete. Included in this summary are investigations mainly presenting a model of liver damage *in vivo*, evaluating parameters of hepatic integrity as a major endpoint, and the administration of melatonin as the primary therapeutic agent. Studies on chronic disease development, aging, investigations on nutritional or dietary changes, exercise-induced stress, remote organ injuries with the liver as a secondary target, as well as investigations on tumor development, cancer progression and liver metastases were excluded.

Based on this extraordinary pool of data, treatment with melatonin appears to be a versatile hepatoprotective strategy in models of experimental liver injury as demonstrated *in vivo* for rats, mice and chicks. There are remarkable variations concerning both the route of melatonin administration, as well as the dose given, the latter ranging a thousand-fold from 100 μg/kg[93,124] to 100 mg/kg[77] melatonin. Only limited data are available on dose-response relationships, and most studies did not include measurements of plasma melatonin levels. Furthermore, it should be mentioned that in some investigations, melatonin was given either as a single dose or repetitively - in some publications for weeks - as a pretreatment, before or while the damage was induced. Unfortunately, not all researchers used melatonin as a therapeutic substance following the infliction of damage, although this would be of high relevance for the evaluation of its clinical use.

Nevertheless, all these studies show similar or even identical results concerning the hepatoprotective effects of treatment with melatonin. Improvements are consistently demonstrated for - but not limited to - parameters of antioxidant enzymes, hepatocellular integrity, interleukin response, NO signaling, and survival.

Antioxidant effects

A strong antioxidant effect of melatonin seems evident as almost all investigators describe that in liver homogenates,

Table 1 Hepatoprotective effects of melatonin in different models of stress

Model	Induction/type	Melatonin treatment	Hepatoprotective effects of melatonin	Species	Ref.
Septic shock	CLP/LPS/LPS + BCG	0.25-60 mg/kg ip/iv/*po* 1-10 ×	hLPO↓, AST/ALT/GGT/ALP/BIL↓, hGSH/hGPx/hSOD/hCAT↑, hNEC↓, hPMN infiltration↓, hTNF-α/hIL-1/hNO↓, 72-h survival rate↑	Rats, mice	[40-49]
Hemorrhagic shock	90 min (MAP 35)/40%	10 mg/kg iv 1 dose	AST/ALT/LDH↓, liver function PDR-ICG↑, hepatic perfusion↑, hNEC↓	Rats	[50-52]
Ischemia/reperfusion	40-60 min ischemia/ischemia + resection	10-20 mg/kg ip/im 1-5 ×	hLPO↓, AST/ALT/LDH↓, hGSH↑, hNEC↓, hMPO↓, hPMN infiltration↓, hTNF-α/hCAS/hAPO/hiNOS↓, 7-d survival rate↑	Rats	[53-62]
Surgical trauma	70% hepatectomy	10 mg/kg per day ip for 7 d	hLPO↓, hGSH↑, histological alterations↓	Rats	[63]
Toxic liver injury	δ-Aminolevulinic acid	10 mg/kg per day ip 7-14 d	hLPO↓, hepatic DNA damage↓	Rats	[64,65]
	Acetaminophen	10-100 mg/kg ip/*po*/sc 1 ×	hLPO↓, AST/ALT↓, hGSH↑, hMPO↓, hNEC↓, 72-h survival rate↑	Mice	[66-68]
	Adriamycin	2-6 mg/kg ip/sc 1-7 ×	hLPO↓, hGSH/hGPx/hCAT↑, hHSP 40/60/70↓	Rats, mice	[69-71]
	Aflatoxins	5-40 mg/kg per day ig/ip for 3-8 wk	hLPO↓, hGSH/hGPx↑, hCAS/hNO↓, hHSP-70↓, hNEC↓	Rats, chicks	[72-76]
	Allyl alcohol	100 mg/kg ip 1 ×	hLPO↓, AST/ALT/LDH↓, hGSH↑, hNEC↓	Rats	[77]
	Arsenic	10 mg/kg ip for 5 d	hLPO↓, hGSH/hSOD/hCAT↑	Rats	[78]
	Cadmium	10-12 mg/kg per day ip/*po* for 3-15 d	hLPO↓, hGSH/hGPx↑, hNEC↓	Rats, mice	[79-82]
	Carbon tetrachloride	10-100 mg/kg ip/sc 1-30 ×	hLPO↓, AST/ALT/ALP/LDH/BIL↓, hGSH/hSOD/hCAT↑, hXO↓, hNO↓, hTNF-α/hIL-1b/hNF-κB↓, hNEC↓	Rats, mice	[77,83-92]
	Cyclophosphamide	100 μg/kg per day *po* for 15 d	hLPO↓, hGSH↑	Mice	[93]
	Cyclosporin A	715 μg/kg per day ip for 14 d	hLPO↓, AST/ALT/GGT↓, hNEC↓	Rats	[94-96]
	Diazepam	5 mg/kg per day sc for 30 d	hLPO↓, hSOD/hGSH↑	Rats	[97]
	Dimethylnitrosamine	50-100 mg/kg per day ip for 14 d	hLPO↓, AST/ALT/ALP/BIL↓, hSOD/hGSH/hGPx/hHO-1↑, hTNF-α/hIL-1b/hIL-6/hNF-κB↓	Rats	[98,99]
	Diquat	20 mg/kg ip 1 ×	ALT↓, hepatic content of F2-isoprostane↓, 24-h survival rate↑	Rats, mice	[100,101]
	Doxorubicin	10 mg/kg sc for 7 d	hLPO↓, GGT/LDH↓	Rats	[102]
	Endosulfan	10 mg/kg ip for 5 d	hLPO↓, AST/ALT/LDH↓, hGSH↑, hMPO↓, hTNF-α/IL-1b↓	Rats	[103]
	Iodine	1 mg/kg per day ip for 14 d	Hepatic content of Schiff's bases↓	Rats	[104]
	Kainic acid	4-10 mg/kg ip 1 ×	Hepatic DNA damage↓	Rats	[105]
	Lead	10-30 mg/kg per day ig for 7-30 d	hLPO↓, hGSH/hGPx/hSOD↑, hNEC↓	Rats	[106,107]
	Methanol	10 mg/kg ip 2 ×	hLPO↓, hGSH/hGPx/hSOD/hCAT↑, hMPO/hNO↓	Rats	[108]
	Methotrexate	10 mg/kg per day ip for 5 d	hLPO↓, hGSH↑, hNEC↓	Rats	[109]
	Mercury-(Ⅱ)	10 mg/kg ip 2 ×	hLPO↓, hGSH↑, hMPO↓	Rats	[110]
	α-Naphthyliso-thiocyanate	10-100 mg/kg ip/*po* 1-4 ×	hLPO↓, AST/ALT/LDH/GGT/ALP/BIL↓, hSOD/hCAT↑, hMPO↓	Rats	[111-114]
	Nodularin	5-15 mg/kg per day ip for 7 d	hGPx/hSOD/hCAT↑	Mice	[115]
	Ochratoxin A	5-20 mg/kg ig/*po* 1-28 ×	hLPO↓, GGT/ALP↓, hGSH/hGPx/hSOD/hCAT↑, hNEC↓	Rats	[116-120]
	Paraquat	1-10 mg/kg ip 5-6 ×	hLPO↓, hGSH↑, LD50 of paraquat↑	Rats	[121,122]
	Phosphine	10 mg/kg ip 1 ×	hLPO↓, hGSH↑	Rats	[123]
	Safrole	0.1-0.2 mg/kg sc 2 ×	Hepatic DNA damage↓	Rats	[124]
	Thioacetamide	3 mg/kg ip 3-5 ×	hLPO↓, AST/ALT/LDH/ammonia↓, hGSH/hCAT↑, hiNOS/hNEC↓	Rats	[125-127]
	Zymosan	5-50 mg/kg ip 1-7 ×	hLPO/hMPO↓	Rats	[128,129]
Cholestasis	Bile-duct ligation	0.5-100 mg/kg per day ip/*po* for 7-13 d	hLPO↓, AST/ALT/GGT/ALP/BIL↓, hGSH/hGPx/hSOD/hCAT↑, hMPO↓, hNO↓, hNEC↓, iron disturbances↓	Rats	[130-140]
Ionizing radiation	Full-body; 0.8-6.0 Gray	5-50 mg/kg ip 1-5 ×	hLPO↓, AST/ALT/GGT↓, hGSH/hSOD/hGPx↑, hMPO/hNO↓, hepatic DNA damage↓	Rats	[141-145]
Malaria	Schistosoma mansoni	10 mg/kg per day ip for 30 d	hLPO↓, AST/ALT↓, hGSH/hSOD↑, 56-d survival rate↑	Mice	[146]

↑: Upregulation/increase/improvement; ↓: Downregulation/decrease/deterioration; ALT: Alanine transaminase; ALP: Alkaline phosphatase; AST: Aspartate transaminase; BCG: Bacillus Calmette-Guérin; BIL: Bilirubin; CLP: Cecal-ligation and puncture; GGT: γ glutamyl transferase; hAPO: Hepatic apoptosis; hCAT: Hepatic catalase; hCAS: Hepatic caspase; hGPx: Hepatic glutathione peroxidase; hGSH: Hepatic glutathione; hHSP: Hepatic heat shock protein; hHO-1: Hepatic heme oxygenase 1; hIL: Hepatic interleukin; hiNOS: Hepatic inducible nitric oxide synthase; hLPO: Hepatic lipid peroxidation; hMPO: Hepatic myeloperoxidase; hNEC: Hepatocellular necrosis; hNF-κB: Nuclear factor κ-light-chain-enhancer of activated B cells; hNO: Hepatic nitric oxide; hPMN: Hepatic polymorphonuclear granulocytes; hSOD: Hepatic superoxide dismutase; hTNF-α: Hepatic tumor necrosis factor α; hXO: Hepatic xanthine oxidase; ig: Intragastrically; im: Intramuscularly; ip: Intraperitoneally; iv: Intravenously; LD: Lethal dose; LDH: Lactate dehydrogenase; LPS: Lipopolysaccharide; MAP: Mean arterial pressure; PDR-ICG: Plasma disappearance rate of indocyanine green; *po*: *Per os*; sc: Subcutaneously.

melatonin strongly attenuated hepatic LPO[40-49,53-99,102,103,106-114,116-123,125-146], usually measured by means of malondialdehyde quantification. Furthermore, melatonin appears to increase the activity and/or expression of hepatic antioxidant enzymes, such as GSH, GPx and SOD, after most types of injury[40-49,53-63,66-93,97-99,103,106-123,125-127,130-146]. Many in-

vestigators also report an increase in hepatic catalase after melatonin treatment[44,71,78,83-85,89,108,111,115,116,118,125,132,135,139].

Hepatocellular integrity

Administration of the pineal hormone appears to reduce the rise in serum enzyme levels of aspartate transaminase, alanine transaminase, lactate dehydrogenase, alkaline phosphatase, γ glutamyl transferase and bilirubin after almost all types of injury, indicating that the extent of cell damage was reduced[40-62,66-68,77,83-92,94-96,98-103,111-114,116-120,125-127,130-146]. This is supported by histopathology results when performed, showing that animals treated with melatonin typically presented with reduced hepatocellular necrosis or attenuated infiltration of polymorphonuclear granulocytes. Reduced hepatic levels of myeloperoxidase further indicate that neutrophil granulocyte infiltration was strongly reduced by the pineal hormone[41,55,67,108,109,111,134,143].

Interleukin response

With respect to interleukin signaling, melatonin was reported to suppress the formation of pro-inflammatory cytokines such as tumor necrosis factor α, interleukin (IL)-1, IL-1β, IL-6, as well as the cellular interleukin response protein, nuclear factor κ-light-chain-enhancer of activated B cells[42,43,53,62,88,99]. This was demonstrated in sepsis and after ischemia/reperfusion, as well as after carbon tetrachloride and dimethylnitrosamine toxicity. Thus, parts of the hepatoprotective actions of the pineal hormone could be based on its suppressive effects on the pro-inflammatory pathway of the immune response.

NO signaling

A large number of studies have investigated the relevance of the NO pathway in the protective effects of melatonin treatment[40,42,43,45,47,49,53,56,57,60,72,73,75,108,125,128,129,142,146]. Melatonin seems to reduce NO release in the vasculature and attenuate the expression of inducible NOS in the liver, as was demonstrated in models of sepsis, ischemia/reperfusion, cholestasis, ionizing radiation, and toxic liver injury with aflatoxins, carbon tetrachloride, methanol, and thioacetamide. As NO reacts with superoxide to form the potentially toxic oxidant peroxynitrite, the reduction in the expression of iNOS may well be another key element in the antioxidant potential of melatonin.

Survival

When investigated, the observed hepatoprotective effects of melatonin were associated with an improvement in survival rate or mean survival time, which was observed in models of sepsis, ischemia/reperfusion, acetaminophen and diquat toxicity, and malaria[41-43,49,53,60,68,101,146].

Taken together, the results from more than 100 experimental studies included here, show convincingly that various regimens of melatonin treatment may be used to reduce hepatic damage in acute liver injury *in vivo*[40-146]. However, this overview is likely to be incomplete: many other studies indicate similar results for chronic disease development and tumor therapy.

So far, only one investigation has been published regarding hepatoprotection by melatonin in humans: in a prospective study, increased survival, attenuated liver damage and reduced immunological activity after transcatheter arterial chemoembolization (TACE) and melatonin treatment were reported in patients with inoperable advanced hepatocellular carcinoma, compared with control patients who underwent TACE but were not given melatonin[147].

Limitations of melatonin

Despite the enormous amount of data supporting the idea of melatonin as a liver protective agent, it should be noted that there are reports which show no hepatoprotective effect of melatonin in a few models of stress. Daniels *et al*[148] were unable to demonstrate any benefit of melatonin administration with respect to carbon tetrachloride-induced liver injury *in vivo*, although ten other studies unanimously showed the value of such a treatment[83-92]. Furthermore, melatonin had no effect on 2-nitropropane-induced LPO in rat liver[149].

Equally interesting and disappointing, melatonin does not appear to be a protective agent with respect to hepatic ethanol toxicity. In a model of acute or chronic ethanol exposure, melatonin administration did not influence hepatic LPO, or GSH and GPx activities in rat[150]. El-Sokkary *et al*[151] demonstrated that administration of ethanol for 30 d did not increase hepatic LPO in the same species. Yet, a recent study showed that melatonin may reduce ethanol-induced liver injury in terms of reduced hepatocellular injury and inflammatory response in a rodent model[152]. As a consequence, further data are required to resolve the issue on whether melatonin may be helpful in reducing ethanol-associated liver damage.

Both positive and negative findings raise the question of how melatonin's intense hepatoprotective potential may be mediated. With respect to this matter, it has been suggested that the activation of membrane-bound melatonin receptors may be an important step in the induction of the antioxidant properties of the pineal hormone[35,36].

HEPATIC MELATONIN RECEPTORS

Melatonin receptors in mammals are classified as membrane-bound, high-affinity G-protein coupled receptors, officially named MT_1 and MT_2 (previous terminology: Mel_{1a} and Mel_{1b}, respectively)[153]. Both receptors are coupled to heterotrimeric G-proteins, and involve signaling through inhibition of cyclic adenosine-monophosphate (cAMP) formation, protein kinase A activity and phosphorylation of cAMP responsive element binding, as well as effects on adenyl cyclases, phospholipase A2 and C, and calcium and potassium channels[154-158]. A third receptor, named MT_3, was demonstrated to be equivalent to intracellular quinone-reductase-2[159]. Non-mammalian species express yet another receptor subtype named Mel_{1c}, which is the first type of melatonin receptor to be discovered[160].

In the liver, the presence of MT_1, MT_2 and MT_3 has been reported in various species[161-171]; Table 2 gives an

Table 2 Melatonin receptors in the liver of various species

Species	MT1	MT2	MT3/QR2	Technique	Ref.
Wistar rat	+	+	NT	RT-PCR	[161,162]
CH3/He mouse	+	+	NT	RT-PCR	[163]
Swiss mouse	+	-	NT	RT-PCR	[164]
Sprague-Dawley rat	-	+	NT	RT-PCR	[165]
Golden rabbitfish	+	+	NT	RT-PCR	[166,167]
European sea bass	-	+	NT	RT-PCR	[168]
Senegalese sole	+	-	NT	RT-PCR	[169]
Syrian hamster	NT	NT	+	Iodine ligand	[170,171]
CD-1 mouse	NT	NT	+	Iodine ligand	[170]
Dog	NT	NT	+	Iodine ligand	[170]
Cynomolgus monkey	NT	NT	+	Iodine ligand	[170]

+: Detected; -: Not detected; MT1: Melatonin receptor type 1; MT2: Melatonin receptor type 2; MT3/QR2: Melatonin receptor type 3/quinone reductase-2; NT: Not tested; RT-PCR: Reverse transcription-polymerase chain reaction.

overview on the current literature demonstrating hepatic melatonin receptor expression or specific iodine ligand binding. So far, there are no original research publications showing proof of hepatic MT_1 or MT_2 receptors in humans. Some evidence points to the possibility that melatonin receptor expression may exhibit circadian variations; this has also been demonstrated for hepatic MT_1 and MT_2[163,166-168].

The physiological significance of hepatic melatonin receptors is mostly unknown. Two studies indicated that hepatic melatonin receptors may be involved in regulating blood glucose[164,172]. Melatonin receptor double knock-out mice do exist, and they appear to have a generally unaltered phenotype. So far, there are no reports showing disadvantages regarding the lack of hepatic melatonin receptors under physiological conditions.

Unfortunately, there are currently no reliable antibodies available for MT_1 and MT_2 receptors[154]. Only a few publications have demonstrated data on the MT_1 or MT_2 protein[162,173]; the results are either non-specific or cannot easily be reproduced. Thus, additional techniques will be required to convincingly demonstrate melatonin receptor protein in the liver.

Nonetheless, our own laboratory was able to generate preliminary results concerning the immunohistochemical distribution of MT_1 in the liver[173]. It appeared that MT_1 was primarily localized in the pericentral area of liver lobules. Due to their metabolic state, pericentral fields of the liver are particularly sensitive to ischemic stress, compared to slightly better oxygenated periportal areas. Thus, this differential distribution of melatonin receptors could provide a way of focusing melatonin receptor-dependent liver protection to areas in need. It is tempting to speculate that this pattern of MT_1 expression might allow the preferential protection of centrolobular hepatocytes.

Further studies, using different techniques or improved antibodies, will be required to support this idea of differentially distributed hepatic melatonin receptors. Thus, the presence and distribution of both melatonin receptor protein subtypes in the liver remain to be determined.

RECEPTOR-MEDIATED ACTIONS OF MELATONIN IN THE LIVER

Only a few studies have analyzed the significance of melatonin receptors in the hepatoprotective effects of melatonin administration *in vivo*[50,51,174]. In a model of hemorrhage and resuscitation, the melatonin receptor antagonist luzindole was able to attenuate the protective effects of melatonin pretreatment and therapy with respect to liver function as measured by plasma disappearance rate of indocyanine green[50,51]. However, not all of the beneficial effects of melatonin were abolished. The use of this antagonist may not clarify all aspects of the effects of melatonin administration, as luzindole itself has been demonstrated to have a strong direct antioxidant potential[175], and to reduce LPO *in vitro*[176].

In the same model of hemorrhagic shock, therapy with the selective melatonin receptor agonist ramelteon improved liver function and hepatic perfusion in rats[174]; this melatonin receptor agonist does not possess any relevant radical scavenging properties[174]. These results point to the possibility that although beneficial, the radical scavenging capacity of melatonin may not be necessary for its protective actions.

This hypothesis is supported by the observation that in other organ systems, the protective potential of melatonin may also be antagonized by luzindole: this antagonist has been reported to abolish the protective capacity of melatonin after myocardial ischemia/reperfusion injury[177], after cyclosporine-A cardiotoxicity[178], in a model of neonatal brain injury[179], and with respect to stress-induced gastric lesions[180].

The following preliminary data from our own research laboratory may have even more impact: in a murine model of sepsis, we were able to demonstrate that the improvements in survival seen after melatonin therapy were not present in melatonin receptor double knock-out mice. This finding indicates once more that membrane-bound melatonin receptors may be responsible for the beneficial effects of melatonin administration.

As a consequence, if (1) no radical scavenging properties are necessary to provide organ protection *via* melatonin receptor activation[174]; (2) the melatonin receptor antagonist luzindole may abolish almost all protective effects of melatonin[177-180]; and (3) the absence of melatonin receptors impedes the protective action of melatonin administration, then it appears reasonable to conclude that melatonin receptors are necessary to mediate at least some of the beneficial effects of the pineal hormone in peripheral organs.

POTENTIAL INFLUENCE ON HEPATIC GENE EXPRESSION

The specific intracellular signal transduction cascade leading to hepatoprotective effects after melatonin receptor activation is presently unknown. However, a number of

hypotheses have been published, suggesting that cAMP responsive element- or estrogen responsive element-containing genes may be regulated by melatonin receptor activation[35,181]. Most certainly, melatonin has a profound influence on hepatocellular gene expression; this has been demonstrated in heat shock protein expression by various investigators[69,73,95]. Our research group was able to present preliminary data showing that melatonin influences different pathways of hepatocellular transcription, including modifications of a variety of heat shock proteins, as well as intense regulation of other membrane-bound receptors and signal transduction factors, in a rat model of hemorrhagic shock[182]. These findings allow the assumption that melatonin therapy may induce beneficial changes with respect to gene transcription in hepatocytes, in the environment of oxidative stress. However, it remains to be determined whether these modifications of hepatic gene expression are indeed mediated by melatonin receptor activation.

FROM BENCH TO BEDSIDE

While the current literature leaves little doubt that melatonin administration may induce hepatoprotective actions[40-146], many questions remain on how this effect may be transduced. The putative signaling cascade, leading from melatonin receptor activation to specific hepatoprotective gene expression profiles, remains to be determined. Based on the evidence available, it appears possible that melatonin receptors mediate the intense protective effects of the pineal hormone in the liver.

To bring this experimental knowledge into clinical use, a pilot study was initiated by Schemmer *et al*[183] in Germany to evaluate the use of melatonin in patients undergoing major liver resections. Should this investigation be successful, this would open the door for yet another important indication for the use of melatonin in human liver surgery: as an adjunct to reduce ischemia/reperfusion injury in liver transplantation. The research group of Freitas and Vairetti has already demonstrated in two studies that melatonin may reduce cold ischemic injury in rat liver[184,185], and suggested that the pineal hormone may be useful in the event of liver transplantation. This idea was supported by Casillas-Ramírez in a review on liver transplantation[186]. Thus, melatonin administration could be beneficial in patients not only to reduce damage to the transplant, but also to serve as a protective agent for the attenuation of reperfusion injury.

Future studies will demonstrate whether melatonin will meet our high expectations not only in the laboratory, but also for our patients. However, the currently available literature allows us to believe that melatonin will successfully continue its way from bench to bedside as a powerful hepatoprotective agent.

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TOPIC HIGHLIGHT

Rene Schmidt, MD, DESA, *Series Editor*

Current protective strategies in liver surgery

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Abstract

During liver resection surgery for cancer or liver transplantation, the liver is subject to ischaemia (reduction in blood flow) followed by reperfusion (restoration of blood flow), which results in liver injury [ischemia-reperfusion (IR) or IR injury]. Modulation of IR injury can be achieved in various ways. These include hypothermia, ischaemic preconditioning (IPC) (brief cycles of ischaemia followed by reperfusion of the organ before the prolonged period of ischaemia i.e. a conditioning response), ischaemic postconditioning (conditioning after the prolonged period of ischaemia but before the reperfusion), pharmacological agents to decrease IR injury, genetic modulation of IR injury, and machine perfusion (pulsatile perfusion). Hypothermia decreases the metabolic functions and the oxygen consumption of organs. Static cold storage in University of Wisconsin solution reduces IR injury and has prolonged organ storage and improved the function of transplanted grafts. There is currently no evidence for any clinical advantage in the use of alternate solutions for static cold storage. Although experimental data from animal models suggest that IPC, ischaemic postconditioning, various pharmacological agents, gene therapy, and machine perfusion decrease IR injury, none of these interventions can be recommended in clinical practice. This is because of the lack of randomized controlled trials assessing the safety and efficacy of ischaemic postconditioning, gene therapy, and machine perfusion. Randomized controlled trials and systematic reviews of randomized controlled trials assessing the safety and efficacy of IPC and various pharmacological agents have demonstrated biochemical or histological improvements but this has not translated to clinical benefit. Further well designed randomized controlled trials are necessary to assess the various new protective strategies in liver resection.

Key words: Liver; Hepatectomy; Liver transplantation; Ischemia-reperfusion injury; Hypothermia; Ischaemic preconditioning

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INTRODUCTION

Approximately 11 000 liver transplantations and an estimated 7000 to 10 000 liver resections are performed every year in US[1-3]. During liver resection and transplantation, the liver is subject to ischaemia (reduction in blood flow). A period of ischaemia is unavoidable in organ transplantation between the time the donor heart stops pumping blood through the circulation and the circulation to the organ is restored in the recipient. When the blood flow is restored (reperfusion), the liver is subjected to further injury. The damage caused by ischemia and then reperfusion in an organ is called ischemia-reperfusion injury (IR injury).

MECHANISM OF IR INJURY

The mechanisms involved in the production of the tissue damage by the IR injury are complex. Overviews of the mechanisms involved in liver IR injury have been described by various authors[4-6]. In simple terms, the sequence of ischaemia followed by reperfusion results in the activation of Kupffer cells (liver macrophages) and polymorphonucleocytes resulting in the production of reactive oxygen species (ROS), cytokines, and adhesion molecule activation leading to liver parenchymal damage.

PROTECTIVE STRATEGIES TO DECREASE LIVER IR INJURY

Modulation of IR injury can be achieved in various ways. These include hypothermia[7,8], ischaemic preconditioning (IPC)[9,10], ischaemic postconditioning[11], pharmacological agents to decrease IR injury[12,13], genetic modulation of IR injury[14], and machine perfusion[7,15]. Systematic reviews of well designed randomized controlled trials (with homogeneity) are currently considered the highest level of evidence to assess the effects of interventions[16]. A well designed randomized controlled trial is the next highest level of evidence[16]. The safety and effectiveness of the different interventions based on randomized controlled trials and systematic reviews of randomized controlled trials in humans is discussed under each of the methods.

Hypothermia

Hypothermia decreases the metabolic functions and the oxygen consumption of organs[17]. Although the organ can be preserved by warm perfusion, hypothermia has been used to decrease IR injury in the transplantation setting for several decades.

Invasive cooling of the donor liver: Ischaemic injury to the liver begins when the donor heart stops pumping blood through the circulation. During the liver retrieval operation, current standard practice involves perfusion of the liver through the aorta with or without perfusion through the portal vein using cold solution[18]. There are no randomized controlled trials comparing hypothermic with normothermic perfusion of the donor organ. Currently, there is evidence from one randomized controlled trial that the incidence of primary graft non-function decreases when double perfusion (aortic and portal vein perfusion) is used compared with single perfusion (aorta alone perfusion) in marginal donors (sub-optimal donors)[18]. In the optimal donor, there is currently no evidence of difference in clinical outcomes between single perfusion and double perfusion[18]. Apart from this comparison of the donor perfusion technique, there is currently no evidence for any difference in the graft or patient survival between the different solutions used for donor perfusion or different pressures used for perfusion[19-21].

Surface cooling of donor liver: There is currently no evidence that surface cooling of the donor liver in addition to invasive cooling by aortic and portal vein perfusion improves liver transplant outcomes.

Static cold storage and storage solutions: After removal of the liver from the cadaver, the liver is stored for a few hours till it can be transplanted to the recipient. This is the time required for the transport of the liver from the retrieval site to the transplant site. During this time, preservation injury occurs. This is because of lack of adequate oxygenation of the tissues. The current standard method for preservation is static cold storage. There have been no randomized controlled trials comparing static cold storage with other methods of organ preservation during transport of the liver. However, static cold storage remains the standard against which all other organ preservation methods can be compared. The introduction of University of Wisconsin (UW) solution in 1988[22] increased the capability of long distance procurement and sharing and decreased the costs associated with long distance procurement by decreasing the preservation injury[23,24]. Although the efficacy of UW solution compared with other solutions available at that time (Collin's solution) was not assessed by randomized controlled trial, the evidence for the benefits of UW solution over Collin's solution was so overwhelming[23,24] that a randomized controlled trial would have been considered unethical. To date, UW solution has remained the gold standard solution against which all other solutions are compared[25]. There is no evidence from randomized controlled trials that any of the other solutions such as Celsior solution or histidine-tryptophan-ketoglutarate solution result in a better graft or patient survival than UW solution[26-30].

Hypothermia in liver resections: While hypothermia has been used as the standard method of decreasing IR injury in liver transplantation, the role of hypothermia as a method of decreasing IR injury in liver resection surgery has not been established. The only randomized controlled trial assessing the impact of in-situ hypothermia in liver resections failed to demonstrate any major clinical benefits of in-situ hypothermia[8].

IPC

IPC is the mechanism by which brief periods of ischaemia followed by reperfusion of the organ results in the ability of the organ to withstand a subsequent prolonged period of ischaemia[31]. Overviews of the mechanisms of IPC have been provided by various authors[5,6,32,33]. Adenosine and nitric oxide play a pivotal role in the IPC response.

IPC can be achieved by a local preconditioning stimulus (direct IPC)[9,10] or by a remote stimulus (remote IPC)[6,34,35]. Remote IPC (RIPC) is the mechanism by which IPC of one vascular bed (area supplied by one artery) protects another vascular bed (area supplied by another artery) from IR injury[35]. The mechanisms involved in RIPC have been reviewed previously[6,34]. Currently, both neural and humoral pathways are believed to be involved in RIPC.

There is experimental evidence that direct IPC and RIPC protects against liver IR injury in the animal model[36-38]. In humans, a systematic review of randomized controlled trials showed that direct IPC decreases the enzyme markers of liver parenchymal injury after liver resections performed under vascular control (i.e. temporary occlusion of blood vessels supplying the liver)[9]. However, this did not translate into any clinical benefit[9]. One randomized controlled trial of remote IPC demonstrated a similar finding i.e. a decrease in the enzyme markers of liver parenchymal injury after liver resections without demonstrating any clinical benefit[39]. There is no evidence for benefit from direct IPC in liver transplantation based on a systematic review of randomized controlled trials[10]. Currently, there are no published randomized controlled trials of RIPC in liver transplantation. Thus, routine IPC (direct IPC or remote IPC) cannot be recommended in either liver resection or transplantation.

Ischaemic postconditioning

As opposed to IPC where the conditioning stimulus is applied prior to the prolonged period of ischaemia, ischaemic postconditioning (IPost) involves the application of the conditioning stimulus (brief intermittent cycles of IR) after the prolonged period of ischaemia but prior to permanent reperfusion i.e. ischaemia followed by conditioning stimulus followed by permanent restoration of blood flow[11]. Overviews of the mechanisms of ischaemic postconditioning have been reviewed previously[40,41]. As with IPC, adenosine and nitric oxide play a pivotal role in ischaemic postconditioning. As in the case of IPC, ischaemic post-conditioning can also be achieved by a local postconditioning stimulus (direct IPost)[11,42-44] or by a remote postconditioning stimulus (RIPost)[45].

In animal models, there is experimental evidence that IPost protects against liver IR injury[42-44]. There are currently no randomized controlled trials of ischaemic postconditioning (direct or remote) in either liver resection or liver transplantation. So, routine ischaemic postconditioning (direct IPost or RIPost) cannot be currently recommended in either liver resection or liver transplantation.

Pharmacologic interventions to decrease IR injury

Various pharmacologic interventions have been attempted with an intention of decreasing IR injury. Considering that ROS and inflammatory mediators play significant roles in IR injury[4-6], pharmacological interventions to neutralise or modulate the pathways using antioxidants and steroids are a subject of significant research[13].

There is experimental evidence that some pharmacological interventions[46,47] protect against liver IR injury in the animal model. In humans, a systematic review of randomized controlled trials assessing the role of pharmacologic interventions in decreasing IR injury after liver resections showed that some interventions such as methyl prednisolone decrease the enzyme markers of liver parenchymal injury after liver resections but without demonstrating evidence of clinical benefit[13]. The role of numerous pharmacological interventions in decreasing IR injury in liver transplantation has been investigated[48-77]. None of the interventions have shown any benefit in graft or patient survival.

Genetic modulation of IR injury

As the molecular mechanisms of IR injury are increasingly understood, more research is being performed on the genetic modulation of the pathways in IR injury both for better understanding of the mechanisms involved in IR injury and for potential therapeutic applications[78]. Experimental evidence to demonstrate the potential role of genetic modulation of liver IR injury exists[14]. There are no randomized clinical trials assessing the impact of genetic modulation of IR injury in liver resections or liver transplantation.

Machine perfusion

Machine perfusion involves pulsatile perfusion of the liver using a machine as opposed to static cold storage. This can be performed by perfusing the liver with a hypothermic perfusate[79] or with a normothermic perfusate[80]. There is experimental evidence in animal models that machine perfusion protects against liver IR injury[80,81]. The safety and efficacy of machine perfusion compared to static cold storage to decrease liver IR injury is yet to be assessed in humans by randomized controlled trials.

DIFFERENCES IN RESULTS BETWEEN ANIMAL MODELS AND HUMAN TRIALS

As discussed above, there are major differences in the results of the role of the different interventions in decreasing liver IR injury between animal models and clinical results. Some possible reasons for this include the lack of fidelity of the model used (i.e. how truly are the results transferable from the model to humans)[82], the use of unvalidated surrogate outcomes, and the use of inadequate sample size in human trials.

FUTURE TRIALS

Future trials of adequate sample size and low risk of bias (low risk of prejudice towards the treatment arm or the control arm)[83] should be performed to decrease the random errors (arriving at wrong conclusions because of pure chance, usually due to inadequate sample size) and systematic errors (arriving at wrong conclusions because of prejudice towards the treatment or the control arm). Measurement of meaningful differences in clinical outcomes requires a large trial. Development and validation of composite outcomes and surrogate outcomes will enable evaluation of the interventions using a smaller sample size.

CONCLUSION

Currently, the only intervention that has shown to be beneficial in the protection of the liver during liver transplantation is hypothermia. In liver resection surgery, there is currently no established intervention targeted at modulating

IR injury that provides any major clinical benefit. However, many new therapies and targets are being discovered. Well designed randomized controlled trials are necessary to assess the new protective strategies in liver resection and liver transplantation.

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ORIGINAL ARTICLE

Promoter polymorphism of MRP1 associated with reduced survival in hepatocellular carcinoma

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Abstract

AIM: To investigate the effect of the G-1666A polymorphism in the multidrug resistance related protein-1 (*MRP1*) on outcome of hepatocellular carcinoma (HCC).

METHODS: A cohort of 162 patients with surgically resected HCC who received no postsurgical treatment until relapse was studied. Genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism analysis. Electrophoretic mobility shift assay (EMSA) was used to evaluate the influence of the G-1666A polymorphism on the binding affinity of the *MRP1* promoter with its putative transcription factors.

RESULTS: Kaplan-Meier analysis showed that patients with GG homologues had a reduced 4-year disease-free survival compared with those carrying at least one A allele (P = 0.011). Multivariate Cox regression analysis indicated that the -1666GG genotype represented an independent predictor of poorer disease-free survival [hazard ratio (HR) = 3.067, 95% confidence interval (CI): 1.587-5.952, P = 0.001], and this trend became worse in men (HR = 3.154, 95% CI: 1.604-6.201, P = 0.001). A similar association was also observed between 4-year overall survival and the polymorphism in men (HR = 3.342, 95% CI: 1.474-7.576, P = 0.004). Moreover, EMSA suggested that the G allele had a stronger binding affinity to nuclear proteins.

CONCLUSION: The *MRP1* -1666GG genotype predicted a worse outcome and was an independent predictor of poor survival in patients with HCC from Southeast China.

Key words: Multidrug resistance related protein-1; Single nucleotide polymorphism; Hepatocellular carcinoma; Prognosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third leading cause of cancer death[1]. Optimal surgical resection is regarded as the best treatment for a curative outcome of HCC. However, long-term survival remains poor because of high rates of tumor recurrence or progression. Substantial effort has been made to identify prognostic factors

that can be used for improving therapeutic regimens and survival prediction. However, only a few factors, such as TNM stage or patient performance status, are consistent predictors, and their accuracy remains limited. Therefore, molecular markers that can accurately predict patient outcome are urgently needed.

The human multidrug resistance protein-1 (MRP1), also known as ABCC1, belongs to the ATP-binding cassette superfamily of cell-surface transport proteins. It participates in the transport of a wide variety of endogenously produced and exogenously administered molecules in an adenosine-triphosphate (ATP)-dependent manner[2,3]. Besides its well-known roles in drug resistance, MRP1 is proposed to contribute to the cellular antioxidative defense system by actively extruding glutathione (GSH)-conjugated xenobiotics and GSH-conjugated metabolites from cells[4]. Recent studies have also revealed that MRP1 is involved in inflammatory reactions, such as, dendritic cell differentiation and function[5]. MRP1 is expressed at moderate levels in most normal tissues, including lung, muscle, and kidney, but is barely detectable in normal liver[6-8]. However, in several liver diseases including HCC, its expression in the basolateral membrane is upregulated, which suggests a significant role for this transport protein during carcinogenesis[8,9].

Single nucleotide polymorphisms (SNPs) in the *MRP1* gene have been extensively studied in the past few years, and several genetic variants in the coding region have been shown to affect the function of MRP1[10-13]. For example, G2168A (Arg723Gln) can affect patients' sensitivity to chemotherapy in ovarian cancer[11]. G1299T (Arg433Ser) confers resistance to doxorubicin by reducing intracellular drug accumulation in HeLa cells that stably express mutant MRP1, whereas the G3173A (Arg1058Gln) variation increases the response to etoposide in HEK293 and CHO-K1 cells[12,13]. Recently, it has been observed that SNPs in the gene promoter can affect expression by disturbing the binding affinity of transcription factors, and are associated with disease prognosis[14]. However, whether SNPs in the *MRP1* promoter region have any clinical significance remains obscure. The expression level of *MRP1* is upregulated in HCC, therefore, we hypothesized that sequence variants in the promoter region potentially affect the expression of the *MRP1* gene and the prognosis of cancer, by modulating the efflux of toxins. To test this hypothesis, we investigated the potential of the *MRP1* G-1666A polymorphism (rs4148330) as a prognostic marker in a cohort of patients with HCC in Guangdong province of Southeast China.

MATERIALS AND METHODS

Study population

The study included 162 patients with HCC at the Cancer Center of Sun Yat-sen University (Guangzhou, China) from 2001 to 2005. All patients underwent hepatectomy as initial therapy, and did not receive chemotherapy or radiotherapy as follow-up treatment before recurrence. All samples were histologically confirmed. After surgical resection, the tissue samples were immediately frozen in liquid nitrogen and then stored at -80℃ until use.

Clinicopathological details and follow-up information were obtained from hospital records. The patients enrolled in the study were residents of Guangdong Province. Infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) was diagnosed when HBV surface antigen or HCV antibody was detected by enzyme linked immunosorbent assay in the serum isolated from peripheral blood. The TNM criteria and the Edmondson and Steiner grading system were used to classify tumor stages and differentiation grades, respectively. Informed consent was obtained from each patient. This study was approved by the Clinical Research Ethics Committee of Sun Yat-sen University Cancer Center.

DNA isolation and genotyping

Total genomic DNA was isolated with a standard protocol that included proteinase digestion, phenol-chloroform extraction, and ethanol precipitation. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was used to detect the genotype. A 160-bp fragment that covered the G-1666A polymorphism was generated using sense primer 5'-GCAACAGCATAACTGGCATT-3' and reverse primer 5'-GAGACCTCCCCCCAATCA-3'. PCR was performed as follows: 20 ng genomic DNA was amplified in a 20-μL reaction mixture that contained 2 mmol/L $MgCl_2$, 0.4 mmol/L dNTPs, 0.2 μmol/L each primer, and 0.5 U *Taq* polymerase (Promega, Madison, WI, USA). After a total of 36 cycles of amplification at an annealing temperature of 58℃, 3 μL PCR products was then incubated overnight at 37℃ with 15 U *Hpa*Ⅱ (MBI Fermentas, Hanover, MD, USA). Digested products were analyzed by 2% agarose gel. PCR fragments that demonstrated altered electrophoretic patterns were purified and characterized by direct DNA sequencing. Results represent two independent experiments.

Cell lines and nuclear protein extraction

Liver cancer cell lines Huh7 and Hep3B were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum in a humidified environment of 37℃ that contained 50 mL/L CO_2. Nuclear protein extracts from Hep3B and Huh7 cells were prepared according to the manufacturer's protocol (NucBuster Protein Extraction Kit; Novagen, Darmstadt, Germany).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed with the Gel Shift Assay System (Promega), according to the manufacturer's instructions. The following oligonucleotides that corresponded to the promoter region of *MRP1* and covered the G-1666A polymorphism were synthesized (underline letters indicate polymorphism): -1666A allele, 5'-GGGGGACCCGGGCCAATAAAAAAATCA-3'; -1666G allele, 5'-GGGGGACCCAGGCCAATAAAAAAATCA-3'; nonspecific (scrambled) oligonucleotide, 5'-GAAGCGGTGACACGGAACATCACGAAA-3'. Oligonucleotides were annealed and end-labeled with [γ-^{32}P]-ATP. Five micrograms of Hep3B or

Huh7 nuclear extracts were added in each binding reaction. For the competition assay, a 10-, 50- or 100-fold molar excess of unlabeled oligonucleotide was added to the binding reaction mixture as a competitor. The products were separated on pre-electrophoresed 5% polyacrylamide gels at 4℃. The gels were then dried at 80℃ for 4 h and exposed to a Storage Phosphor Screen (Amersham Bioscience, Sunnyvale, CA, USA), which was subsequently read with a Typhoon Phosphor Imager (Amersham Bioscience). The putative transcription factors that recognized the sequences that overlapped the G-1666A site were predicted with Alibaba2.1 (http://www.gene-regulation.com/pub/programs/alibaba2/index.html) and the transcription element search software (TESS, http://www.cbil.upenn.edu/cgi-bin/tess/tess).

Statistical analysis

The χ^2 and Fisher's exact tests were used for the analysis of the relationship between the genotypes and clinicopathological characteristics. Disease-free survival (DFS) was calculated from the day of surgery to either relapse or death without relapse, and it was censored only for patients who were alive and recurrence-free at the last follow-up. Overall survival (OS) was measured from the date of hepatectomy to the time of death or the last follow-up. Survival curves were obtained by the Kaplan-Meier method, and the statistical significance of the differences in survival among subgroups was evaluated with the log-rank test. The Cox proportional hazards model was employed to assess the independent prognostic values of the polymorphisms. Statistical analyses were all performed with SPSS software package (version 13.0; SPSS, Inc., Chicago, IL, USA). All statistical tests were two-sided, and $P < 0.05$ was considered to be statistically significant.

RESULTS

Patient characteristics and genotype

Demographic and clinicopathological characteristics of the 162 patients with HCC are summarized in Table 1. The mean age at first diagnosis of HCC was 48 years. Consistent with our previous study[15], most patients showed excessive γ-glutamyl transpeptidase and α-fetoprotein, along with liver cirrhosis, and > 80% of the enrolled patients were infected with HBV (140/161, 87.0%), which implicated HBV infection as a leading cause of HCC in Southeast China. In contrast, only a small number of patients were infected with HCV.

Genotyping was performed by PCR-RFLP. A 160-bp *MRP1* promoter region that covered the G-1666A variant was digested with *Hpa*Ⅱ. After full digestion of the amplified PCR products, those from AA homozygotes still existed as a single 160-bp fragment, whereas those from the GG homozygotes had been divided into two fragments of 71 bp and 89 bp, respectively. The allele frequency of patients with HCC was 0.61 for *MRP1* -1666A and 0.39 for -1666G. However, no significant correlations were found between the nucleotide variants and clinical variables (data not shown).

Table 1 Physiological characteristics of hepatocellular carcinoma patients (n = 162)

	n (%)
Sex	
Female	15 (9.3)
Male	147 (90.7)
Age (yr)	
< 48	77 (47.5)
⩾ 48	85 (52.5)
HBV infection[1]	
-	21 (13.0)
+	140 (86.4)
HCV infection	
-	158 (97.5)
+	4 (2.5)
GGT (U/L)[2]	
< 50	45 (27.8)
50-99	48 (29.6)
⩾ 100	67 (41.4)
AFP (ng/mL)	
< 20	53 (32.7)
20-399	45 (27.8)
⩾ 400	64 (39.5)
Tumor size (cm)	
< 5	53 (32.7)
⩾ 5	109 (67.3)
Ascites[3]	
-	148 (91.4)
+	14 (8.6)
Cirrhosis	
Total	19 (11.7)
Mild	80 (49.4)
Moderate	49 (30.2)
Severe	14 (8.6)
Edmondson grade	
Ⅰ	11 (6.8)
Ⅱ	71 (43.8)
Ⅲ	77 (47.5)
Ⅳ	3 (1.9)
TNM stage	
Ⅰ	106 (65.4)
Ⅱ	7 (4.3)
Ⅲ	49 (30.3)
G-1666A genotype	
AA	55 (34.0)
AG	89 (54.9)
GG	18 (11.1)

[1](-) absence, (+) presence, one case unconfirmed; [2]Two cases unconfirmed; [3](-) absence, (+) presence. HBV: Hepatitis B virus; HCV: Hepatitis C virus; GGT: γ-glutamyl transpeptidase; AFP: α-fetoprotein.

Association analysis between the G-1666A polymorphism and survival

Growing evidence suggests that SNPs are closely related to the risk and outcome of cancer[14,16,17]. To investigate the impact of the G-1666A polymorphism on the prognosis of patients with HCC, we next analyzed the 4-year DFS and OS of patients with different genotypes. A significant correlation between the -1666 polymorphism and postoperative survival was found. The mean survival times of patients with the AA, AG and GG genotypes were 30.4 ± 18.2, 30.7 ± 17.4 and 24.8 ± 17.3 mo, respectively. The survival curves showed that the 4-year rate of DFS among patients who carried GG decreased significantly compared with those who carried the AA or AG allele (P = 0.031,

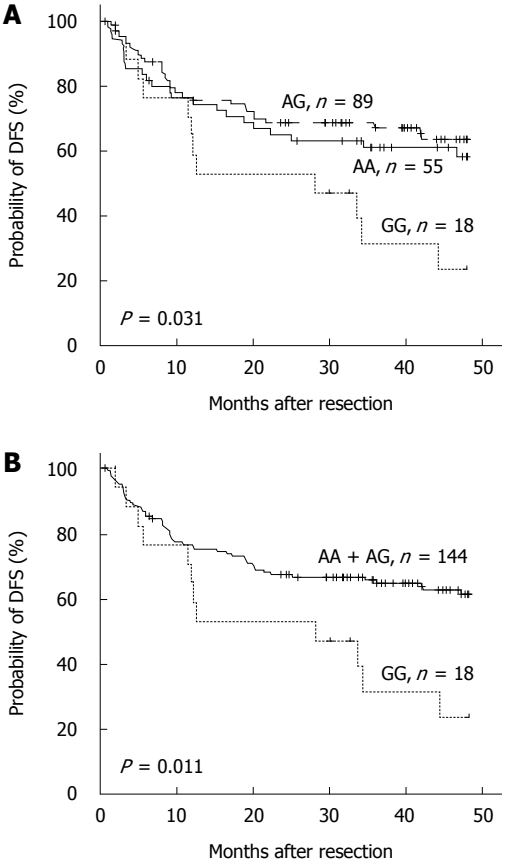

Figure 1 Kaplan-Meier disease-free survival curves for hepatocellular carcinoma patients who carried different *multidrug resistance related protein-1* -1666 genotypes. A: Comparison between three genotypes; B: GG genotype compared with the other two genotypes. Log-rank *P* values are indicated. Tick marks represent censored data. DFS: Disease-free survival.

Figure 1A). Moreover, if the patients with AA and AG genotypes were combined, the discrepancy became more obvious ($P = 0.011$, Figure 1B). Further analysis revealed a similar, albeit non-significant, trend between the -1666 polymorphism and 4-year OS (Table 2). Multivariate Cox proportional hazard analysis was then performed, and the variables that showed significance by univariate analysis were adopted as covariates (Table 2). The results revealed that the *MRP1* G-1666A polymorphism was an independent prognostic factor for 4-year DFS [hazard ratio (HR) = 3.067, 95% confidence interval (CI): 1.587-5.952, $P = 0.001$, Table 3].

One of the key features of HCC is the much higher incidence in men than in women[1]. In our study cohort, the male/female ratio was 9.8:1. Further stratification of the patients by sex revealed an even more pronounced association of the -1666GG genotype with poorer survival ($P < 0.05$, Figure 2). Multivariate analysis suggested that the -1666GG genotype was an especially powerful independent prognostic factor of 4-year DFS (HR = 3.154, 95% CI: 1.604-6.201, $P = 0.001$) and OS (HR = 3.342, 95% CI: 1.474-7.576, $P = 0.004$) in the men with HCC (Table 3).

Influence of the G-1666A polymorphism on the affinity of binding with nuclear proteins

We performed EMSA to evaluate the influence of the

Table 2 Determination of prognostic factors for disease-free survival and overall survival of patients with hepatocellular carcinoma, by univariate analysis ($n = 162$)

Variable	DFS		OS	
	HR (95% CI)	*P*[a]	HR (95% CI)	*P*[a]
Sex				
Female	1		1	
Male	4.231 (1.035-17.299)	0.045	7.522 (1.041-54.337)	0.045
GGT (U/L)[1]				
< 50	1		1	
50-99	2.064 (0.920-4.632)	0.079	1.771 (0.734-4.275)	0.203
⩾ 100	3.639 (1.761-7.519)	0.001	3.728 (1.734-8.014)	0.001
AFP (ng/mL)				
< 20	1		1	
20-399	1.852 (0.954-3.595)	0.068	2.004 (0.989-4.061)	0.054
⩾ 400	1.997 (1.070-3.725)	0.030	2.102 (1.079-4.094)	0.029
Tumor size (cm)				
< 5	1		1	
⩾ 5	2.230 (1.233-4.030)	0.008	2.089 (1.126-3.875)	0.019
Ascites[2]				
-	1		1	
+	2.562 (1.301-5.044)	0.007	2.81 (1.375-5.741)	0.005
Cirrhosis				
No	1		1	
Mild	2.005 (0.710-5.661)	0.189	2.505 (0.763-8.225)	0.130
Moderate	1.832 (0.623-5.387)	0.271	2.287 (0.670-7.806)	0.187
Severe	3.230 (0.993-10.505)	0.051	4.796 (1.297-17.738)	0.019
TNM stage				
Ⅰ	1		1	
Ⅱ + Ⅲ	3.165 (1.940-5.163)	< 0.001	3.424 (2.038-5.752)	< 0.001
Genotypes				
AA	1		1	
AG	0.830 (0.479-1.439)	0.507	0.818 (0.463-1.447)	0.490
GG	1.988 (0.982-4.024)	0.056	1.491 (0.682-3.258)	0.317
AA + AG	1		1	
GG	2.223 (1.185-4.172)	0.013	1.678 (0.822-3.422)	0.155

[a]Hazard ratio (HR) and *P* values were calculated using univariate Cox regression. $P < 0.05$ was considered to indicate statistical significance; [1]Two cases unconfirmed; [2](+) presence, (-) absence. DFS: Disease-free survival; OS: Overall survival; CI: Confidence interval; GGT: γ-glutamyl transpeptidase; AFP: α-fetoprotein.

G-1666A polymorphism on the binding affinity of the *MRP1* promoter with putative transcription factors. The radiolabeled -1666G probe showed strong DNA-protein binding ability in the presence of nuclear proteins extracted from the Hep3B cell line, whereas the -1666A probe barely showed any interaction (Figure 3A, lane 2 and lane 10, respectively). In order to assess the binding specificity and the differences in binding affinity between the G and A alleles, competition assays were performed with unlabeled -1666A and -1666G oligonucleotides. A 50-fold excess of unlabeled -1666A oligonucleotides only partially disrupted the binding of the radiolabeled -1666G probe with nuclear extracts (Figure 3A, lane 7 and Figure 3B, lane 6), whereas this amount of unlabeled -1666G oligonucleotides almost completely abolished the binding (Figure 3A and B, lane 4). In contrast, a non-specific competitor had no effect (Figure 3B, lane 8). Similar results were obtained when using the nuclear extracts from Huh7 cells (data not shown). These data suggested that the G-1666A polymorphism could affect the binding affinity of the *MRP1* promoter with transcription factors, and

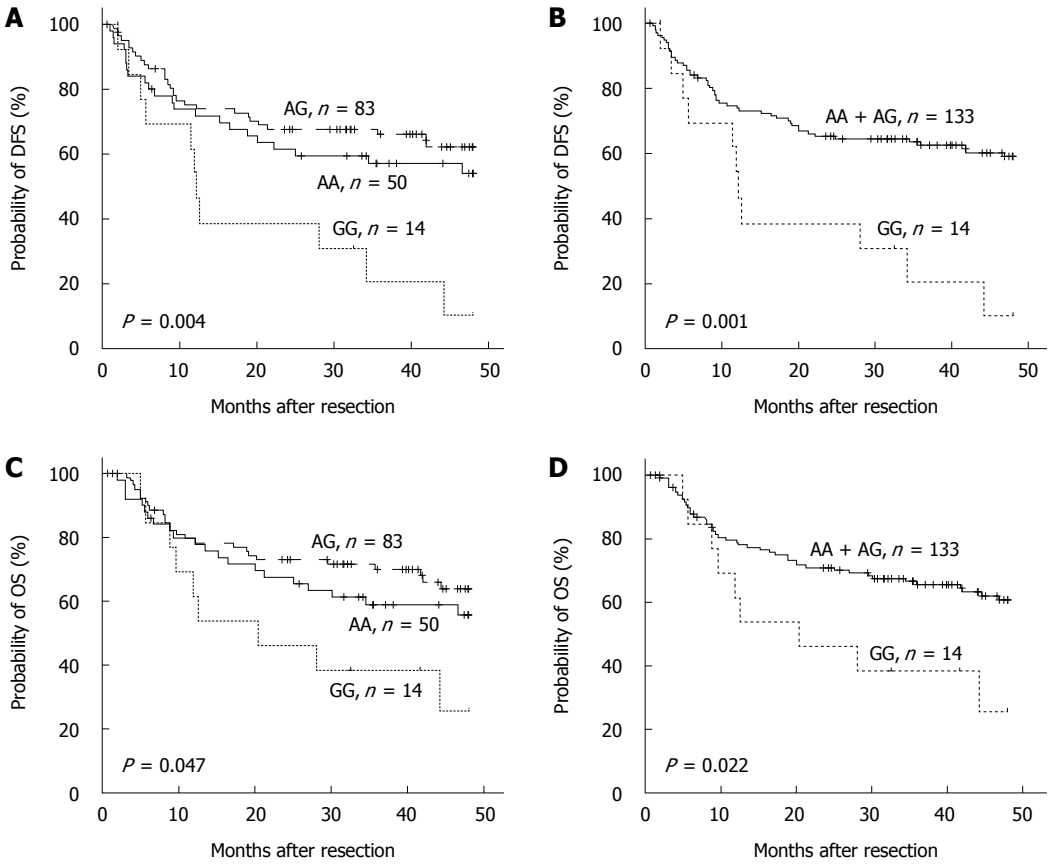

Figure 2 Kaplan-Meier curves for disease-free survival (A and B) and overall survival (C and D) for male patients with hepatocellular carcinoma and different *multidrug resistance related protein-1* -1666 genotypes. A: Comparison of disease-free survival (DFS) between three genotypes; B: AA and AG grouped together and compared to GG genotype; C: Comparison of overall survival (OS) between three genotypes; D: OS of AA and AG genotypes compared with GG genotype. Log-rank *P* values are indicated. Tick marks represent censored data.

Table 3 Multivariate analysis for prognostic value of *multidrug resistance related protein-1* G-1666A polymorphism in patients with hepatocellular carcinoma

Genotypes	DFS[1]		OS[2]	
	HR (95% CI)	*P*[a]	HR (95% CI)	*P*[a]
All (*n* = 162)				
AA+AG	1			
GG	3.067 (1.587-5.952)	0.001		
Men (*n* = 147)				
AA + AG	1		1	
GG	3.154 (1.604-6.201)	0.001	3.342 (1.474-7.576)	0.004

[a]Hazard ratio (HR) and *P* values were calculated using multivariate Cox regression. $P < 0.05$ was considered to indicate statistical significance; [1]Multivariate analysis of disease-free survival (DFS) in all patients was adjusted for sex, γ-glutamyl transpeptidase (GGT), α-fetoprotein (AFP), tumor size, ascites, and TNM stage; in male patients, it was adjusted for GGT, tumor size, and TNM stage; [2]Multivariate analysis of OS in male patients was adjusted for GGT, tumor size, ascites, cirrhosis, and TNM stage. OS: Overall survival; CI: Confidence interval.

that the G allele had a stronger binding affinity than the A allele.

DISCUSSION

The multidrug resistance protein family transports a wide range of physiological substrates and diverse therapeutic agents. In the past decade, much effort has been focused on MRP1-mediated drug resistance[18,19]; and emerging evidence indicates that SNPs within the *MRP1* gene have prognostic value in predicting the response to chemotherapy in different cancers[11,20]. Notably, MRP1 takes part in the transport of aflatoxin B1, a well-know human liver carcinogen that can induce a characteristic mutation in *p53* at codon 249[21,22]; and previous studies have shown that *p53* mutations are significantly associated with a poor prognosis for patients with HCC[15,23]. In addition, MRP1 also plays important roles in cellular antioxidant defense and immune cell function[4,5]. These observations and the upregulated expression level of *MRP1* gene in several liver diseases, including HCC, suggest the possibility that this protein is involved in tumorigenesis and progression[8,9]. Our present study examined the role of the *MRP1* G-1666A polymorphism as a prognostic factor in patients with HCC who were treated only with curative surgery, and proved that the GG genotype was an independent predictor of poor survival, especially in men with HCC. Furthermore, specific binding of nuclear proteins to G allele was found, which suggested a difference in transcription activity between different genotypes.

In the present study, there were 147 men (90.7%) and 15 women with HCC (9.3%), with a male-to-female ratio

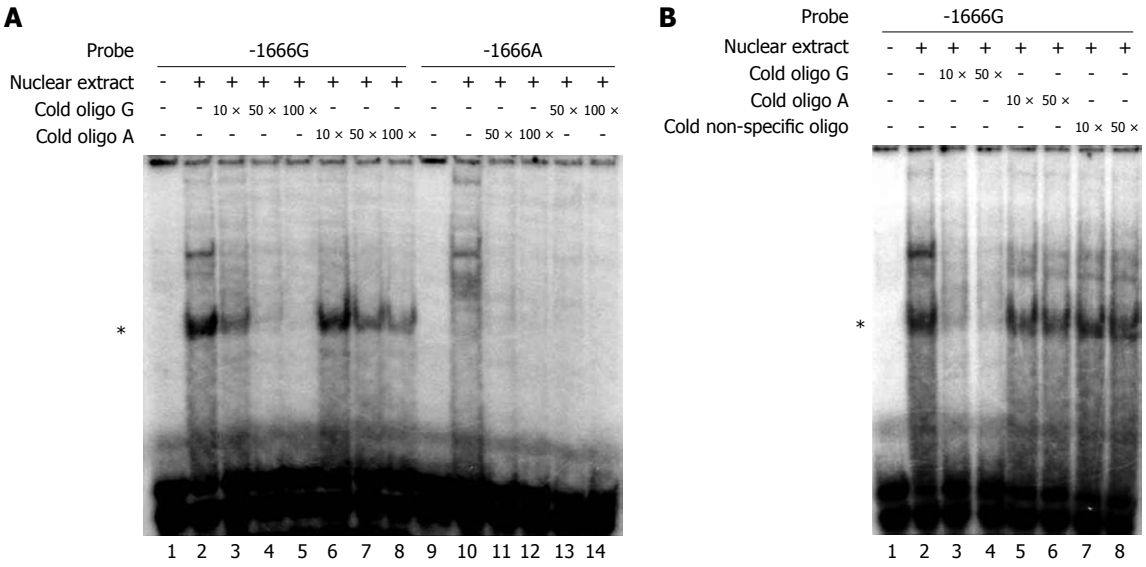

Figure 3 Electrophoretic mobility shift assay of the *multidrug resistance related protein-1* promoter region that contained the G-1666A site. A: Analysis was performed in the presence (+) or absence (-) of Hep3B nuclear extract. Each binding reaction contained γ-^{32}P-labeled -1666G (lanes 2-8) or -1666A (lanes 10-14) probes. A 10-, 50-, or 100-fold (as indicated) excess of unlabeled (cold) -1666A or G oligonucleotides (lanes 6-8, 11, and 12 or 3-5, 13, and 14) were included in the binding reactions as specific competitors. Labeled oligonucleotides incubated without the nuclear extracts were included as negative controls (lanes 1 and 9); B: In the present of Hep3B nuclear extract, 10- or 50-fold more excess of unlabeled -1666G oligonucleotides (lanes 3 and 4) or -1666A oligonucleotides (lanes 5 and 6) or non-specific oligonucleotides (lanes 7 and 8) were used as competitors. Lane 1 was the negative control. Lane 2 indicated the labeled -1666G oligonucleotides incubated with the nuclear extracts only. The asterisks indicated the DNA-protein complex.

of 9.8:1. We observed a remarkably significant association of the *MRP1* G-1666A polymorphism with 4-year OS in men with HCC, but not in the entire cohort. This phenomenon might result from the interaction between the polymorphism and sexual hormones during carcinogenesis, which has been demonstrated in the example of *MDM2* SNP309[24]. Therefore, the correlation between the *MRP1* G-1666A polymorphism and the survival of women with HCC requires further investigation to generate a definite conclusion.

SNPs in the promoter region of a gene can potentially alter the affinity of interactions between DNA and nuclear proteins and, in turn, affect the efficiency of transcription. We found that the G allele of the *MRP1* G-1666A polymorphism had a stronger binding affinity for nuclear proteins in hepatoma cells than the A allele had. This finding accords with our presumption that the G-1666A polymorphism might dominate the pumping ability of MRP1 by affecting the expression of the protein. Although a G-1666A polymorphism located 1.5 kb upstream of the core promoter of *MRP1*, and two major regulatory domains had already been found in tandem upstream of the core promoter[25], recent studies have revealed that distal regions (enhancer or suppressor) can influence gene transcription through physical association with the transcription start site[26]. Furthermore, allele G of the G-260C polymorphism could lead to lower activity of the *MRP1* promoter in cell lines, which suggests that nucleotide variants in the *MRP1* gene account, in part, for inter-individual variations and population differences in cellular efflux[27]. These data suggest that the G-1666A polymorphism functions as a distal element through the folding of the DNA strand.

The three transcription factors Sp1, NF-1 and CTF were predicted to bind to the promoter region, including the G-1666A site, by Alibaba2.1 software, but only the Sp1 consensus motif could partially disrupt the DNA-protein binding in a competition assay, whereas the other two did not show significant influence on the binding (data not shown). Sp1 antibody failed to reveal any super-shift band when added to the EMSA reaction complex (data not shown), which suggested that Sp1 binding to the cis-element, including the G-1666A site, required an interaction between Sp1 and other nuclear proteins. Future work will be required to identify such nuclear proteins.

In summary, our present study shows that the *MRP1* G-1666A polymorphism is an independent prognostic factor for patients with HCC, which implies a role for MRP1 in tumor progression. Clearly, much more work remains to be done to confirm our findings and overcome the limitations in our work before this SNP can be used as a marker for poor outcome in HCC.

ACKNOWLEDGMENTS

The authors are grateful to Professor Shi-Mei Zhuang for her advice and guidance on this paper.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third leading cause of cancer death, and long-term survival remains poor because of high rates of tumor recurrence or progression. Therefore, markers that can be used for improving therapeutic regimens and survival prediction are urgently needed.

Research frontiers

The finding that human multidrug resistance protein-1 (MRP1) is expressed

in unusually large amounts in HCC suggests it has a role in the growth and progression of this cancer. Expression of MRP1 is affected by the genetic sequence in its promoter region, therefore, the authors of this study examined the potential of different sequences (polymorphisms) in the *MRP1* promoter to serve as indicators of prognosis and outcome in patients with HCC.

Innovations and breakthroughs

Recent studies have demonstrated that mutations within the *MRP1* gene have value in predicting the response to chemotherapy in different cancers, but the clinical significance of such mutations in the *MRP1* promoter for patients with HCC is unknown. This is believed to be the first study to identify a polymorphism in the promoter of *MRP1* that is an independent prognostic factor for 4-year overall survival in men with HCC. The correlation between the *MRP1* polymorphism and the survival of women with HCC requires further investigation. The authors also demonstrated that the polymorphism altered the affinity of nuclear proteins for the DNA in the HCC cells, which might explain the mechanism by which the expression of MRP1 was reducing.

Applications

The genetic sequence identified in this study can be used to test tissue samples from patients with HCC to help predict their outcome after therapy. This information can be used to guide treatment decisions and improve therapeutic regimens for individual patients.

Terminology

MRP1 is one of a family of proteins found on the surface of cells. These proteins transport a wide variety of substances and can contribute to resistance to chemotherapy by transporting anticancer drugs out of cancer cells. The promoter region of a gene is a sequence of nucleotides that regulates whether and how much of a protein is synthesized from that gene.

Peer review

This is a study of an important area of cancer genomics. The authors found that a single nucleotide polymorphism of *MRP1* promoter region is an independent prognostic factor for HCC patients. The study design was well-organized and they reached a conclusion by making full use of the data.

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ORIGINAL ARTICLE

Impaired PI3K/Akt signal pathway and hepatocellular injury in high-fat fed rats

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Abstract

AIM: To determine whether mitochondrial dysfunction resulting from high-fat diet is related to impairment of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt, also known as PKB) pathway.

METHODS: Rat models of nonalcoholic fatty liver were established by high-fat diet feeding. The expression of total and phosphorylated P13K and Akt proteins in hepatocytes was determined by Western blotting. Degree of fat accumulation in liver was measured by hepatic triglyceride. Mitochondrial number and size were determined using quantitative morphometric analysis under transmission electron microscope. The permeability of the outer mitochondrial membrane was assessed by determining the potential gradient across this membrane.

RESULTS: After Wistar rats were fed with high-fat diet for 16 wk, their hepatocytes displayed an accumulation of fat (103.1 ± 12.6 *vs* 421.5 ± 19.7, $P < 0.01$), deformed mitochondria (9.0% ± 4.3% *vs* 83.0% ± 10.9%, $P < 0.05$), and a reduction in the mitochondrial membrane potential (389.385% ± 18.612% *vs* 249.121% ± 13.526%, $P < 0.05$). In addition, the expression of the phosphorylated P13K and Akt proteins in hepatocytes was reduced, as was the expression of the anti-apoptotic protein Bcl-2, while expression of the pro-apoptotic protein caspase-3 was increased. When animals were treated with pharmacological inhibitors of P13K or Akt, instead of high-fat diet, a similar pattern of hepatocellular fat accumulation, mitochondrial impairment, and change in the levels of PI3K, Akt, Bcl-2 was observed.

CONCLUSION: High-fat diet appears to inhibit the PI3K/Akt signaling pathway, which may lead to hepatocellular injury through activation of the mitochondrial membrane pathway of apoptosis.

Key words: Nonalcoholic fatty liver; Phosphatidylinositol 3-kinase/protein kinase B signaling pathway; Mitochondria; B-cell lymphoma gene 2; Caspase-3

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is caused by

triglyceride (TG) accumulation within the liver and can either be a benign self-limiting state or a condition associated with steatohepatitis (NASH), which may develop to fibrosis, cirrhosis and liver failure[1,2]. Triacylglycerol formation in the hepatocytes may also be cytotoxic to hepatocytes[3,4]. Multiple lines of evidence support the role of intrahepatic fat in causing hepatic insulin resistance. Hepatic insulin resistance is considered to be the fundamental mechanism in the prevalence and progression of the disease. It is also a critical component in the development of NAFLD, which is characterized by a marked reduction in the activity of the insulin signaling pathway[5].

In the presence of insulin, the insulin receptor normally phosphorylates insulin receptor substrate (IRS) proteins, which are linked to the activation of several signaling pathways, including the metabolic phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt, also known as PKB) pathway. Phosphatidylinositol 3-kinase (PI3K) and its downstream effector Akt regulate a diverse array of cellular events, including survival and apoptosis of a number of cell types[6].

Hepatocyte apoptosis is a key histologic feature of NAFLD, and correlates with progressive inflammation and fibrosis[7]. The molecular pathways leading to hepatocyte apoptosis are not fully defined; however, mitochondrial dysfunction is an important element in the pathogenesis of NAFLD[8]. Recent evidence showed that mitochondria participate in the regulation of both cell proliferation and death, including apoptosis[9], and are thus potential mediators of the PI3K/Akt signaling pathway. Although multiple lines of evidence have suggested a close linkage between insulin-induced signaling and mitochondrial functions[10], the potential relationship between the PI3K/Akt signaling pathway and the mitochondrial abnormalities that underlie NAFLD remain unclear.

The present study was undertaken to investigate the relationship between mitochondria impairment and the activity of the PI3K/Akt signaling pathway during the development of NAFLD.

MATERIALS AND METHODS

Animal groups and diet

Male Wistar rats, 12 wk old were obtained from Harbin Medical University.

Laboratories (stock No. 002207). All experiments and animal care complied with the guidelines for the humane treatment of animals set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences, Harbin Medical University. At 6 wk of age, the 60 mice were randomly divided into four groups: (1) Normal control (NC) group; (2) NC plus the PI3K inhibitor LY294002 (NC + LY, 15 μg/kg daily injected *via* the tail CA 440206, Calbiochem); (3) NC plus the AKT inhibitor 1-L-6-hydroxymethyl-chiro-inositol2-(R)-2-O-methyl-3-O-octadecylcarbonate (NC + AI, 20 μg/kg daily *via* tail injection CA124005, Calbiochem); and (4) High-fat diet (HFD). The normal control rats were fed a commercial rat diet (7%-10% fat, 68%-70% carbohydrates, 18%-20% protein, 1%-2% vitamins and minerals; 210 kcal/100 g per day) for 16 wk, while rats in the treatment group (HFD group) were fed a high-fat diet (40% fat, 38%-40% carbohydrates, 18%-20% protein, 1%-2% vitamins and minerals; 210 kcal/100 g per day) for the same period of time.

Calculation of metabolic index and resistance index

Blood samples from the retro-orbital sinus were collected before and after the treatment. Rats were fasted overnight before the collection of the blood samples. Plasma insulin was determined using ELISA. Insulin resistance was evaluated using a homeostasis model assessment of insulin resistance (HOMA). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyltransferase (GGT) levels were measured using spectrophotometric assay kits (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Insulin resistance was assessed by computing insulin resistant index (HOMA-IR). The formula used was as follows: HOMA-IR = Insulin (μg/L) × glucose (mmol/L)/22.5.

Measurement of hepatic TG

The liver (100 mg wet tissue) was homogenized in an ice-cold 0.05% butylhydroxytoluene solution. After lipids were extracted from the liver according to the method of Folch *et al*[11], TG content in each sample was measured with a commercial assay kit (Wako Pure Chemical Industries, Osaka, Japan CA 290-63701).

Isolation of hepatocytes

Hepatocytes were isolated from the liver (20-25 mg) of each mouse by the collagenase perfusion method. Each liver was pre-perfused at 37℃ with buffer containing 100 mmol/L HEPES (pH 7.4), 143 mmol/L NaCl, and 7 mmol/L KCl, and then perfused with buffer containing 0.05% collagenase and 5 mmol/L $CaCl_2$. Following digestion, the liver was dispersed in the perfusion solution and incubated in the perfusion buffer at 37℃ for an additional 5 min. The dispersed cell suspension was then filtered through a nylon mesh and centrifuged at 100 × *g* for 3 min at 25℃. The resulting cell pellets were resuspended in the hepatocyte medium, and cell viability was then determined using a trypan-blue-exclusion test.

Measurement of mitochondrial membrane potential of hepatocytes

The integrity of the inner mitochondrial membrane was assessed by determining the potential gradient across this membrane. Rhodamine 123 (Rh123) powder was dissolved in methanol and stored at -20℃ as a 1 g/L solution, which was diluted to 5 mg/L with phosphate buffered solution (PBS) before each experiment. Hepatocytes (1×10^6) were washed three times with PBS that had been preheated to 4℃. They were then resuspended in 300 mL PBS, incubating with Rh123 (final concentration 2.5 mg/L) for 1 h at 37℃, and then filtered through a 200-mesh screen. Approximately 10000 cells were measured using a FACS Calibur flow cy-

tometer (BD Biosciences, San Diego, CA, USA) using Cell Quest software (a maximum absorbing wave length 590 nm, an excitation wave length 488 nm) (BD Biosciences). Rh123 and tetramethylrhodamineethylester (TMRE) were purchased from Invitrogen (Karlsruhe, Germany).

Electron microscopy

For transmission electron microscopy, small liver fragments were fixed in 4% glutaraldehyde and then processed using standard methods. Sections were viewed under microscope by a pathologist (Dr. Chang H, Department of Pathology, Harbin Medical University). Mitochondrial number and size were determined using quantitative morphometric analysis under transmission electron microscope (Model HB601UX, Vacuum Generators, Hastings, United Kingdom).

Western blotting

Ten μg protein was subjected to SDS-PAGE (10% acrylamide gel) and then transferred to a PVDF membrane for 2 h (120 V) using a Bio-Rad Mini Trans Blot electrophoretic transfer unit (Bio-Rad, Marnes-la-Coquette, France). The membranes were blocked for nonspecific binding with 5% nonfat dry milk and then probed with the specific primary antibodies (Abcam, CA ab74136, ab63566, ab79360, ab8805, 1:1000 dilution) at 4℃ overnight. After 3 washes with TBS-T, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA SC2030). Separated proteins were visualized by an ECL kit (GE Healthcare Life Sciences, CA RPN2135) and light emission was captured on X-ray film (GE Healthcare). Intensities of the respective bands were examined by densitometric analysis (Scion Image Analyst program).

Statistical analysis

Results were presented as mean ± SE and were analyzed using one-way analysis of variance followed by the Bonferroni multiple comparisons test. All tests were 2-sided, and $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using INSTAT version 3 (Graph Pad Software, San Diego, CA, USA).

RESULTS

Comparison of body and liver weight, biochemical parameters, insulin resistant index among four groups

Compared with normal control groups, the mean serum transaminase, hepatic TG content, body and liver weight, insulin resistant index were all increased in PI3K inhibitor, Akt inhibitor and high-fat groups ($P < 0.05$, Table 1). Hepatocytes could be damaged after PI3K/Akt pathway signal transduction was blocked, but blocked PI3K/Akt pathway signal transduction could lead to TG accumulation in liver to liver weight gain and insulin resistance.

Table 1 Weights of the liver and serum levels of biochemical parameters in high-fat fed rats

	NC	NC + LY	NC + AI	HFD
Tissue weight				
Liver (g)	1.55 ± 0.14	1.99 ± 0.32[a]	1.89 ± 0.43[a]	3.98 ± 0.64[a]
Serum				
TG (mg/dL)	183.8 ± 70.4	396.7 ± 72.3	376.7 ± 68.4	589 ± 98.4[b]
ALT (U/L)	34 ± 3.1	86 ± 5.58[a]	89 ± 5.2[a]	207 ± 35.5[b]
AST (U/L)	36 ± 3.4	88 ± 6.1	99 ± 5.2[a]	187 ± 35.5[b]
GGT (U/L)	52 ± 6.4	48 ± 3.9	62 ± 4.4	232 ± 67.8[b]
Glucose (mmol/L)	5.1 ± 0.4	6.1 ± 0.5	5.7 ± 0.4	7.1 ± 71.3[a]
Insulin (mIu)	4.7 ± 0.7	14.9 ± 2.0[b]	15.0 ± 2.9[b]	21.0 ± 3.8[b]
Liver				
IRI (HOMA-IR)	1.06	4.03[a]	3.8[a]	6.6[b]
TG (mg/g)	103. 1 ± 12.6	324.6 ± 13.4	2336.8 ± 11.6	421.5 ± 19.7[b]

Rats were fed and subjected to each measurement. Data are presented as the mean ± SD. [a]$P < 0.05$, [b]$P < 0.01$ *vs* rats fed with normal diets. NC: Normal control; NC + LY: NC plus the PI3K blocker LY294002; NC + AI: NC plus the AKT blocker; HFD: High-fat diets for 16 wk ($n = 10^{-12}$); ALT: Alanine aminotransferase; AST: Aspartate aminotransferase. GGT: γ-glutamyltransferase; TG: Triglycerides; IRI (HOMA-IR): Insulin resistant index.

Expression of PI3K and Akt protein in hepatocytes in high-fat fed mice

To further investigate whether the PI3K/Akt pathway mediates injury of hepatocytes, we measured the protein expression levels of total and phosphorylated PI3K and Akt in the four groups. The protein expression levels of total PI3K and Akt showed no significant difference among the four groups (Figure 1). But compared with the normal control groups, the expression levels of pPI3K and pAkt markedly decreased in the high-fat group. Moreover, in PI3K inhibitor groups, neither pPI3K nor pAkt was expressed. In Akt inhibitor groups, pAkt showed no expression, and pPI3K had no significant difference compared with the normal control group (Figure 1). These results suggested that fat mass accumulation in the liver may lead to decreased expression of pPI3K and pAkt in fatty liver induced by high fat.

Fat accumulation in the liver and PI3K/Akt pathway

To investigate further whether the fat accumulation in the liver was involved in PI3K/Akt pathway, TG content in each sample was measured in the four groups. The results showed that TG content of liver was elevated in high-fat diet, PI3K and Akt inhibitor groups.

Ultrastructure of hepatocellular mitochondria and PI3K/Akt pathway

To determine whether the PI3K pathway has an anti-apoptotic effect in liver cells, we compared the mitochondrial morphology in the four groups (Figure 2A). Electron microscopy revealed that the hepatocytes of the control group rats were rich in mitochondria, the shape and size of mitochondria were normal and had few lipid droplets. In contrast, many mitochondria from rats in the high-fat group were enlarged and showed morphological changes, including a rarefied matrix and large lipid droplet. In the high-fat group, 83.0% ± 10.9% of mitochondria had abnormal morphology, compared to 9.0% ± 4.3% in the control group ($P < 0.05$) (Figure 2B). The changes

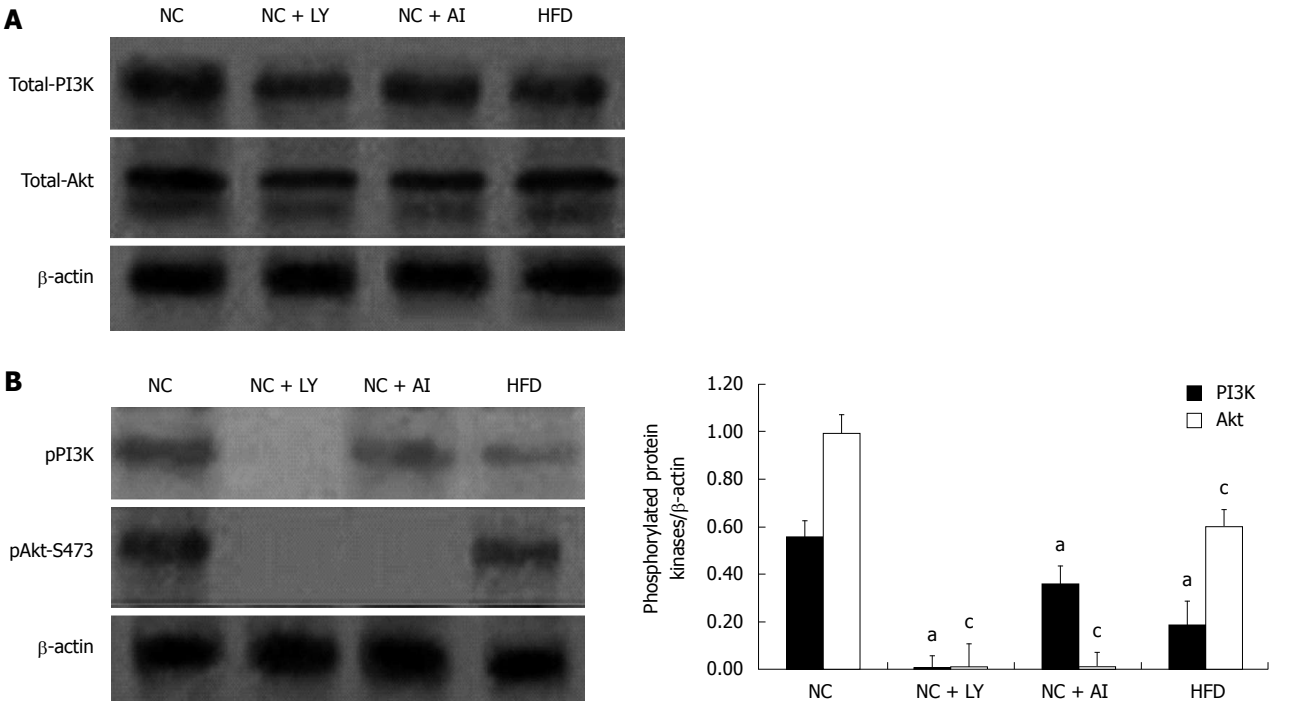

Figure 1 Western blotting analysis of phosphatidylinositol 3-kinase and protein kinase B in hepatocytes. A: Western blotting analysis of total phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt). The protein expression levels of total PI3K and Akt showed no significant difference among the four groups; B: Western blotting analysis of phosphorylated PI3K and Akt. Left: In high-fat fed groups (HFD), the protein expression levels of both phosphorylated PI3K and Akt decreased compared with the normal control (NC) group. In PI3K blocker groups (NC + LY), pPI3K and pAkt showed no expression. In AKT blocker groups (NC + AI), pPI3K was normally expressed and pAkt showed no expression. Right: Band density values are compared with those of the β-actin loading control. $^{a,c}P < 0.05$ *vs* NC group.

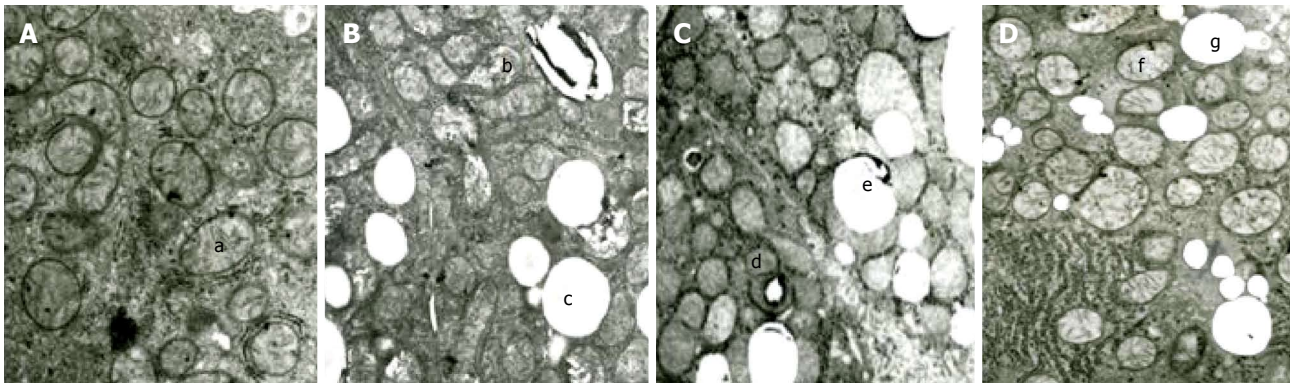

Figure 2 Ultrastructural changes in hepatocellular mitochondria. A: Mitochondria (a) in normal diet group; B: Enlarged mitochondria (b) and lipid droplet (c) in high-fat diet group; C and D: Animals treated with either phosphatidylinositol 3-kinase blocker (C) or protein kinase B blocker (D) showed similar changes in mitochondrial shape, size (d, f) and lipid droplet (e, g) to those of the high-fat group. Magnification × 18 000.

of mitochondria in the PI3K-inhibitor and Akt- inhibitor groups were similar to those of the high- fat group, although they differed significantly from the high-fat group in the PI3K and Akt inhibitor groups (Figure 2C and D). This indicated that PI3K/Akt pathway blocking could affect ultrastructural changes of hepatocellular mitochondria. The change was similar to that resulting from a high-fat diet.

Hepatocellular mitochondria function and PI3K/Akt pathway

To investigate whether PI3K/Akt signaling is associated with an increase in the permeability of the outer mitochondrial membrane, we measured the mean fluorescence intensity (MFI) of Rh123 on the mitochondrial membrane in animals of the four groups. The MFI was 249.121% ± 13.526% in the high-fat group, and 389.385% ± 18.612% in the control group, indicating that there was a significant decrease in the mitochondrial membrane potential in the high-fat group. The PI3K and Akt inhibitor groups showed similar decreases in the mitochondrial membrane potential (211.326% ± 12.114% and 214.326% ± 13.321%, respectively) compared with the control group (Figure 3). These results suggested that mitochondrial function was impaired to the same degree in the high-fat and both inhibitor groups, strongly sug-

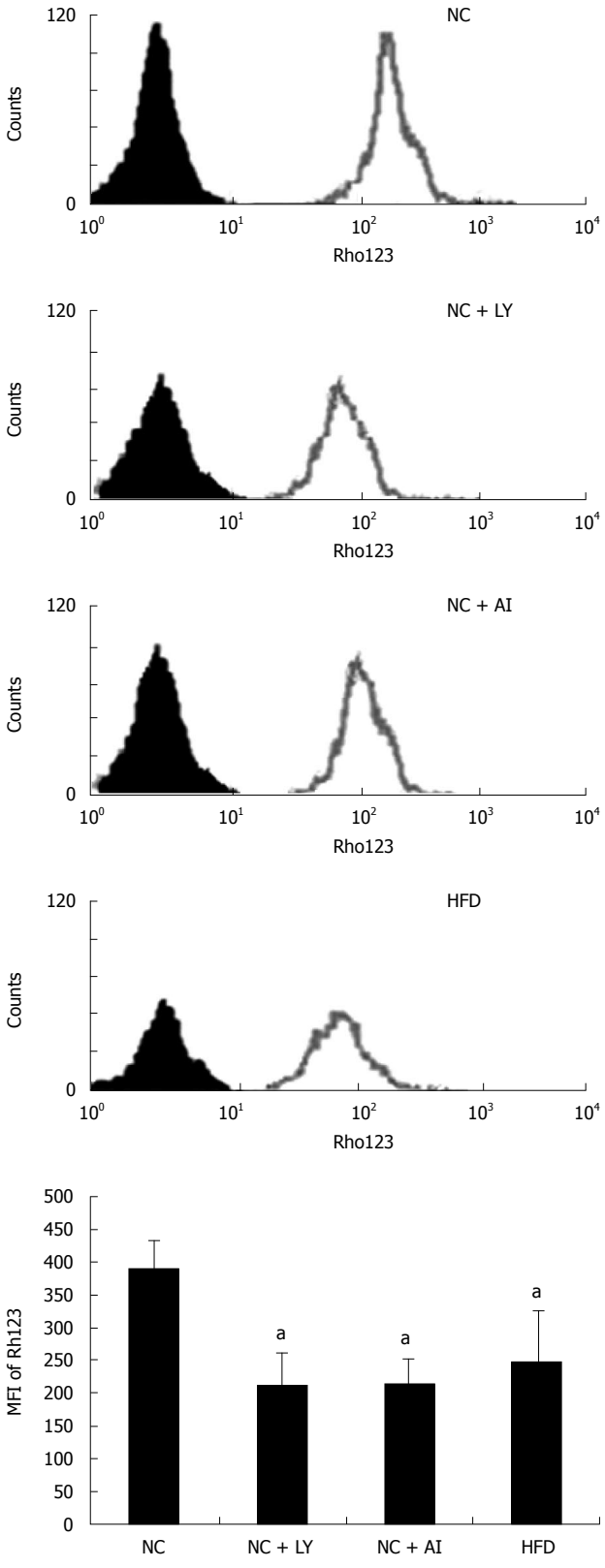

Figure 3 Changes in the membrane potential of hepatocyte mitochondria. Fluorescence intensity (MFI) of Rh123 was 389.385% ± 18.612% in the normal control (NC) group, but decreased to 268.326% ± 13.526% in the high-fat group (HFD); and in PI3K and Akt blocker groups, MFI had a very similar change (211.326% ± 12.114%, 214.326% ± 13.321%, respectively), indicating a decrease in the mitochondrial membrane potential (Dym) in high-fat, PI3K and Akt blocker groups. $^{a}P < 0.05$ *vs* NC group.

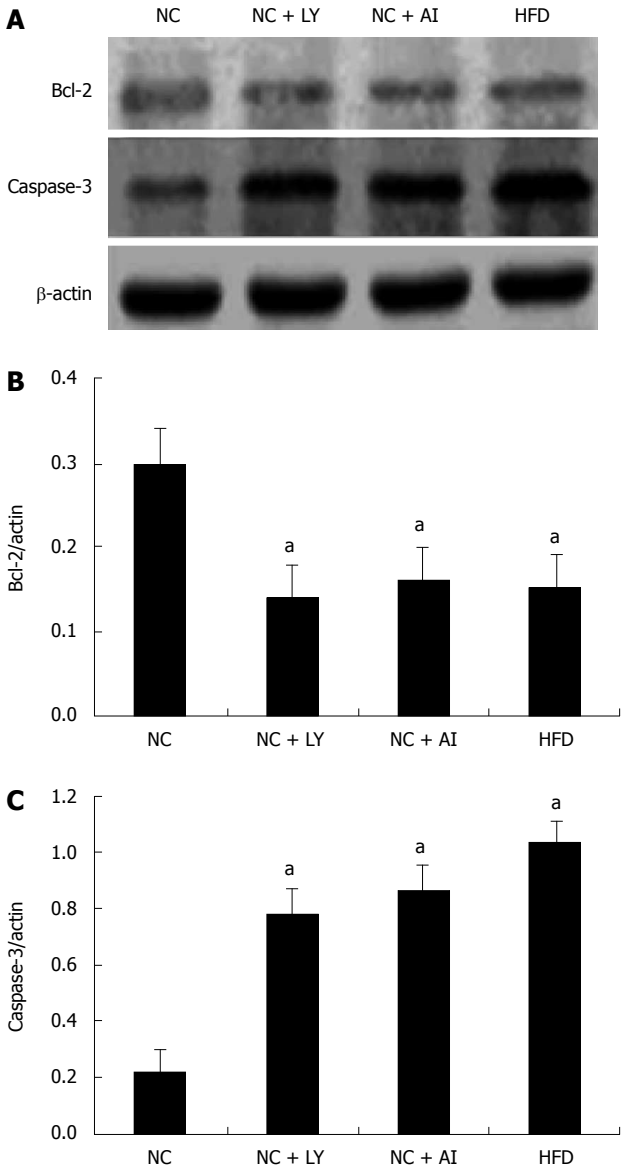

Figure 4 Western blotting analysis of caspase-3 and Bcl-2 in hepatocytes. A: Compared with control values [normal control (NC) group, lane 1], the expression levels of Bcl-2 protein decreased in the phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt)-blocker and high-fat groups (lanes 2-4). In contrast, caspase-3 expression was equally elevated in each of the three treatment groups compared with the control value; B, C: Band density values are compared with those of the β-actin loading control. $^{a}P < 0.05$ *vs* NC group.

gesting that the PI3K/Akt signal transduction pathway is associated with permeabilization of the outer mitochondrial membrane.

Apoptotic genes and PI3K/Akt pathway

One way in which changes in the PI3K/Akt signaling pathway might have induced mitochondrial apoptosis was through the effects of the apoptosis-related proteins Bcl-2 and caspase-3. To test this theory, we measured the expression level of these two proteins using Western blottings (Figure 4). We found that Bcl-2 expression showed a similar decrease in the high-fat, P13K-inhibitor, and

Akt-inhibitor groups (by 64%, 61% and 62%, respectively) compared to the control group, Conversely, the level of caspase-3 increased by a similar margin in the 3 experimental groups (42%, 31% and 29.5%, respectively) compared to the control group. This result demonstrated that there may be a link between the PI3K/Akt signaling pathway and the mitochondrial pathway of apoptosis in rats treated with high-fat, P13K inhibitor, or Akt inhibitor.

DISCUSSION

In this study, we attempted to investigate whether the PI3K/Akt pathway could mediate mitochondrial impairment during the development of NAFLD, and whether this might explain why hepatic insulin resistance is critical to the development of this disease. We found that hepatocytes of rats fed a high-fat diet accumulated fat and developed deformed mitochondria, a decreased mitochondrial membrane potential, and decreased expression of PI3K and Akt proteins. Unexpectedly, we found that blocking the PI3K/Akt pathway with either a PI3K or Akt inhibitor led to hepatocellular fat accumulation and mitochondrial impairment indistinguishable from that of high-fat fed rats. These findings suggest that signals transduced by the PI3K/Akt pathway are involved in the pathogenesis of NAFLD.

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling cascade is an important component of insulin signaling in normal tissues, where it mediates glucose uptake and homeostasis, as well as being an important regulator of cell survival in numerous cell types[12]. In this study, we found that both the insulin resistance index (HOMA-IR) and serum levels of hepatocellular enzymes were significantly increased in response to a high-fat diet, suggesting that insulin resistance and hepatocyte damage may coexist in our high-fat experimental model.

Mitochondrial dysfunction is known to play a central role in the hepatocyte damage of NAFLD[13]. Although our current experiment confirmed that mitochondrial dysfunction is associated with progression of liver pathological changes, the mechanisms initiating mitochondrial dysfunction in this disease are unknown. Specifically, it is not clear whether mitochondrial damage and changes in insulin signal transduction are directly related to the pathophysiology of nonalcoholic fatty liver induced by high fat. A number of studies have indicated that insulin can stimulate mitochondrial biogenesis[14] and alter mitochondrial morphology in obese, insulin-resistant and type 2 diabetic individuals[15]. In the current study, we found that a high-fat diet led to an increase in the proportion of morphologically abnormal mitochondria as well as an increase in the insulin resistance index. Similarly, less marked results were seen with treatment of the P13K- or Akt inhibitors, suggesting that changes in the PI3K/Akt signal transduction pathway may mediate these changes in mitochondria morphology.

The transmembrane potential (Dym) of mitochondria is known to play a crucial role in their normal function[16]. We found that the mitochondrial transmembrane potential

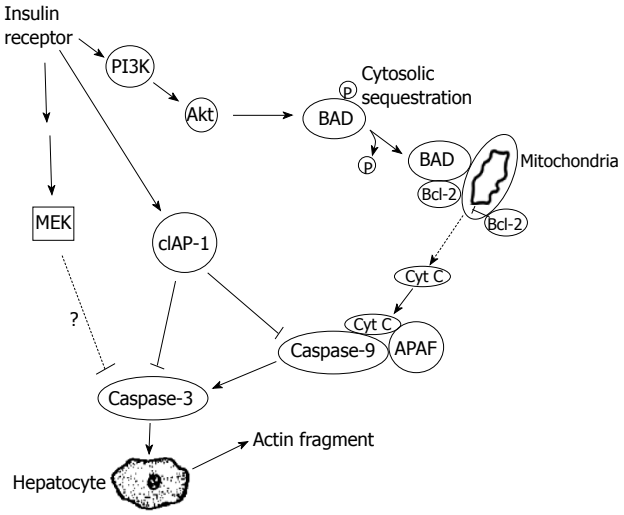

Figure 5 Simulation diagram of the relationship between insulin signaling pathway and hepatocyte apoptosis. PI3K: Phosphatidylinositol 3-kinase; Akt: Protein kinase B; BAD: Bcl2-associated agonist of cell death; MEK: Mitogen-activated protein kinase kinase; cIAP-1: Cell inhibitors of apoptosis protein-1; Cyt C: Cytochrome c; APAF: Apoptotic peptidase activating factor.

in the high-fat group, as well as those treated with either the PI3K- or AKT inhibitor, was significantly lower than control values, further implicating the P13K/Akt pathway in mitochondrial dysfunction.

Our results have confirmed some previous findings by Mehta *et al*[17], who reported that hepatic steatosis is frequently associated with obesity, type 2 diabetes, and hyperlipidemia, with insulin resistance being a key pathogenic factor in NAFLD and mitochondrial damage being characteristic of the disease. However, the current study is the first to implicate the PI3K/Akt signal transduction pathway in the morphological and functional changes in hepatocyte mitochondria, as well as insulin resistance induced by high fat.

Recent evidence suggests that hepatocellular injury in a number of liver diseases is accompanied by activation of the apoptotic pathways[18]. We found morphological (deformed mitochondria), and functional abnormalities (a reduction in the mitochondrial membrane potential) in hepatocyte mitochondria consistent with apoptosis in high-fat, PI3K and Akt inhibitor groups. We also examined the expression of two proteins in the Bcl-2 family of anti-apoptotic proteins. Proteins in this family are known to regulate apoptosis at peri-mitochondrial sites. The PI3K/Akt signaling pathway has been shown to have an anti-apoptotic effect by activating Bcl-2 to inhibit the apoptotic mediator caspase-3[19]. We found that, in rats fed a high-fat diet, expression of PI3K, phosphorylated Akt, and Bcl-2 decreased, but the expression of caspase-3 increased, suggesting a mechanism by which apoptosis may be triggered in NAFLD. To further support a role for the P13K pathway in mediating apoptosis in NAFLD, we found that the P13K- and Akt inhibitors led to a similar decrease in Bcl-2 expression and a significant increase in caspase-3 expression (Figure 5).

Apoptosis is a process of active cellular self-destruc-

tion that requires the expression of specific genes including those of the Bcl-2 gene family[20]. Of these, Bax, Bad and Bak promote cell death, whereas Bcl-2 and Bcl-xL inhibit apoptosis and promote cell survival[21]. The results of a recent study suggest that caspase-3 can cause permeabilization of cells, with the help of pro-apoptotic Bcl-2 proteins. Until recently, the prevailing view has been that caspase-3 activation represents the apex of the caspase cascade within the mitochondrial apoptotic pathway.

Some Bcl-2 family members located on the mitochondrial membrane have been shown to be able to alter the permeability of the mitochondrial membrane and trigger the activation of caspases[22]. Programmed cell death might thus be activated *via* a membrane bound pathway, in which the signal is initiated at the mitochondrion[23]. Pro-apoptotic compounds are normally sequestered in the intermembrane space[24]. When the permeability of the outer mitochondrial membrane increases, these proteins are released into the cytosol, forming the apoptosome and subsequently activating caspase-3[25]. The results of the current study indicate that not only high fat, but also blockage of the PI3K/Akt pathway signal, lead to an increase in the permeability of the hepatic mitochondrial membrane, implicating this pathway in the apoptotic mechanisms triggered by a high-fat diet. All these results lead us to propose a disease model in which the PI3K/Akt signal transduction pathway induces apoptosis *via* a Bcl-2/caspase-3 mitochondrial-dependent pathway, in which phosphorylation of Bad results in targeting of Bcl-xL to the mitochondrial membrane, where Bcl-2 interacts with and inactivates anti-apoptotic Bcl-2 proteins, thereby inducing apoptosis[26].

In conclusion, the present study suggests that fat accumulation in the liver may impair PI3K/Akt pathway signal transduction and thereby activate the mitochondrial membrane pathway of apoptosis, leading to hepatocyte damage.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is caused by triglyceride (TG) accumulation within the liver, which may progress to fibrosis, cirrhosis and liver failure. Mitochondrial dysfunction which caused hepatocyte apoptosis is an important element in the pathogenesis of NAFLD. Hepatic insulin resistance is also a critical component in the development of NAFLD, which is characterized by a marked reduction in the activity of the insulin signaling pathway, including the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt, also known as PKB) pathway. But the potential relationship between the PI3K/Akt signaling pathway and the mitochondrial abnormalities that underlie NAFLD remain unclear.

Research frontiers

Recent reports have highlighted the insulin-induced signaling pathways in various cells. In the presence of insulin, the insulin receptor normally phosphorylates insulin receptor substrate (IRS) proteins, which are linked to the activation of several signaling pathways, including the PI3K/Akt pathway. PI3K and its downstream effector Akt regulate a diverse array of cellular events, including survival and apoptosis of a number of cell types. The mitochondrial impairment during the development of NAFLD is also an area of intense research. Mitochondrial dysfunction is an important element in the pathogenesis of NAFLD, mainly hepatocyte apoptosis. Recent evidence showed that mitochondria participates in the regulation of both cell proliferation and death, including apoptosis, and are thus potential mediators of the PI3K/Akt signaling pathway.

Innovations and breakthroughs

This is the first study to report the relationship between mitochondria impairment and the activity of the PI3K/Akt signaling pathway during the development of NAFLD, which suggests that fat accumulation in the liver may impair PI3K/Akt pathway signal transduction and thereby activate the mitochondrial membrane pathway of apoptosis, leading to hepatocyte damage.

Applications

The results of this study indicated that the PI3K/Akt pathway may mediate mitochondrial impairment during the development of NAFLD, and this might explain why hepatic insulin resistance is critical to the development of this disease.

Terminology

Insulin signaling pathways, including the metabolic PI3K/Akt pathway. PI3K and its downstream effector Akt regulate a diverse array of cellular events, including survival and apoptosis of a number of cell types. Mitochondrial impairment, an important element in the pathogenesis of NAFLD. Mitochondria participates in the regulation of both cell proliferation and death, including apoptosis, and are thus potential mediators of the PI3K/Akt signaling pathway.

Peer review

This is a well written paper with well thought out, well-controlled data. The paper evaluates the effect of a high-fat diet and correlates it with similar findings seen with blocking the P13K or Akt inhibitors, and thereby implicated this pathway as the mechanism for high-fat diet hepatic injury.

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BRIEF ARTICLE

High prevalence of nonalcoholic fatty liver in patients with idiopathic venous thromboembolism

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Abstract

AIM: To assess the prevalence of nonalcoholic fatty liver disease (NAFLD) in patients with idiopathic venous thromboembolism (VTE).

METHODS: In a case-control study, after excluding subjects with well-consolidated risk factors for VTE, idiopathic VTE was documented in 138 consecutive patients who were referred to our department. Two hundred and seventy-six healthy sex/age/body-mass-index-matched subjects, without any clinical/instrumental evidence of VTE, served as controls. All underwent a clinical/laboratory/ultrasound assessment for the presence of metabolic syndrome and NAFLD.

RESULTS: NAFLD was detected in 112/138 cases (81%) and in 84/276 controls (30%) [risk ratio: 2.7, 95% confidence interval (CI): 2.2-3.2, $P < 0.0001$]. Metabolic syndrome and smoking habit were more prevalent in patients with idiopathic VTE. The high prevalence of NAFLD in VTE was also confirmed after adjustment for inherited thrombophilia. NAFLD was clearly predicted by VTE (odds ratio: 1.8, 95% CI: 1.2-2.7, $P < 0.0001$).

CONCLUSION: NAFLD was independently associated with idiopathic VTE.

Key words: Thromboembolism; Metabolic syndrome; Nonalcoholic fatty liver disease

Peer reviewer: Astrid van der Velde, PhD, Team Wetenschap, Netherlands Heart Foundation, PO Box 300, 2501 CH, The Hague, The Netherlands

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INTRODUCTION

Venous thromboembolism (VTE) has an annual incidence of 1-2 events/1000 people in the general population, and is considered to be an emerging health problem[1,2]. Arterial and venous thromboses have been historically considered as distinct entities due to thrombus composition and different response to antiplatelet or anticoagulant drugs[3]. Metabolic syndrome (MS), which affects > 20% of the whole population[4,5], increases cardiovascular risk[6] by a blood hypercoagulability-related mechanism. This phenomenon is the result of abnormally high plasma levels of plasminogen activator inhibitor-1 (PAI-1), fibrinogen and factors Ⅶ, Ⅷ and von Willebrand, as well intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1[7-12]. Indeed, antiphospholipid syndrome and hyperhomocysteinemia predispose to venous and

cardiovascular events[13-15]. Ageno *et al*[16] have reported an association between idiopathic VTE and MS, which has been confirmed by Ay *et al*[17]. Arterial hypertension is a cardiovascular risk factor for VTE[18,19]. In addition, type 2 diabetes is associated with several coagulation and fibrinolysis alterations that lead to a procoagulant, thrombogenic predisposition, and is likely to have a significant impact on VTE occurrence[20]. Abdominal obesity is currently accepted as an independent risk factor for VTE[21].

Nonalcoholic fatty liver disease (NAFLD) has been strictly associated with MS[22]. Insulin resistance is reckoned to be the major mechanism. NAFLD refers to a wide spectrum of liver damage, which ranges from simple steatosis to nonalcoholic steatohepatitis, advanced fibrosis and cirrhosis.

In a series of patients with idiopathic VTE, we tried to assess the prevalence of NAFLD, further expression of MS, comparing the data with those achieved in control subjects.

MATERIALS AND METHODS

Inclusion criteria

One hundred and thirty-eight patients with recent (< 6 mo) objective diagnosis of idiopathic VTE were enrolled in the study. One hundred and twenty patients had deep vein thrombosis (DVT), of which, 21 were associated with superficial vein thrombosis, nine were suffering from isolated pulmonary embolism (PE), and 16 with PE plus DVT. DVT was confirmed by Doppler ultrasonography (DUS). PE was documented by computed tomography.

VTE was defined as idiopathic in the absence of pregnancy or puerperium, known active malignancies, recent (< 3 mo) surgery or trauma, fracture, immobilization, lack of prophylaxis, acute medical disease, use of oral contraceptives, long-distance travel, a history of VTE or repeated birth loss. In contrast, when at least one of the previous risk factors was present, VTE was defined as secondary and the patients were excluded from the study.

As many as 276 healthy sex/age/body mass index (BMI)-matched subjects served as controls. In all of them, exclusion of DVT was based on clinical examination, use of D-dimer testing, and clinical pretest probability and, in some uncertain cases, by two DUS examinations within 1 wk of each other.

Exclusion criteria

Cases and controls who presented with unstable medical conditions were excluded. Other exclusion criteria were a history of infectious chronic diseases including hepatitis B and C, autoimmune and storage diseases, drug-induced hepatic steatosis, and prior use of medication known to affect inflammation, glucose metabolism or blood lipids. Alcohol abuse was ruled out, according to the DSM-IV diagnostic criteria, by means of screening tests such as MAST (Michigan Alcohol Screening Test) and CAGE (Cut down, Annoyed, Guilty, and Eye opener), as well as random tests for blood alcohol concentration and the use of a surrogate marker, e.g. mean corpuscular volume. Patients on antihypertensive therapy maintained a balanced medical regimen throughout the study.

Clinical, laboratory and imaging data

Sex, age, BMI, waist circumference, history of symptomatic atherosclerosis (i.e. ischemic stroke, transient ischemic attack, acute myocardial infarction, angina, intermittent claudication), arterial hypertension or use of antihypertensive drugs, diabetes mellitus or use of antidiabetic drugs, hyperlipidemia or use of statins or clofibrate, smoking habit (daily consumption of $\geqslant$ 1 cigarette), current use of heparin, oral anticoagulant or antiplatelet drugs were recorded. Subsequently, all patients underwent liver ultrasound (US), measurement of blood pressure, fasting glucose, transaminases and γ-glutamyl transferase activity, high-density lipoprotein (HDL) cholesterol and triglyceride levels. MS was diagnosed by the presence of at least three criteria (National Cholesterol Education Adult Treatment Panel Ⅲ) on the basis of abdominal obesity (waist circumference > 102 cm for men and > 88 cm for women), triglycerides $\geqslant$ 150 mg/dL, HDL-cholesterol < 40 mg/dL for men and < 50 mg/dL for women, blood pressure $\geqslant$ 130 mmHg and/or $\geqslant$ 85 mmHg, and fasting glucose $\geqslant$ 100 mg/dL. Obesity was recognized as a BMI $\geqslant$ 30.

The classification of "bright liver" or hepatic steatosis grade was based on the following scale of hyperechogenity at US: 0 = absent, 1 = light, 2 = moderate, 3 = severe, pointing out the difference between the densities of the liver and the right kidney[23]. Diagnostic criteria for DVT were observation of an intraluminal venous thrombus, loss of compressibility, and lack of flow at DUS.

Statistical analysis

We observed how many times the event of interest, i.e. NAFLD occurred in the experimental group or cases (VTE) and in controls. Statistical confidence was increased by taking two controls per case. The RR and 95% CI was the ratio of the proportions of cases with a positive outcome in the two groups. Patients' clinical characteristics were compared using Student's *t* test (continuous variables) and the χ^2 test (dichotomous variables). A logistic regression (stepwise model) was adopted, in which NAFLD was the dependent variable and sex, anthropometric parameters (BMI, waist circumference), metabolic features (serum HDL-cholesterol, triglycerides and glucose), systolic blood pressure, diastolic blood pressure, smoking habit and finally VTE were employed as independent variables. MS as entity was not considered in prediction, to avoid multicollinearity. The same tool (enter method) was carried out to predict VTE presence the by US grade of steatosis. Statistical analysis was performed with MedCalc® 11.2.

RESULTS

The mean age in the cases and controls was 41.8 ± 13.0 and 43.4 ± 15.7 years, and the mean BMI in the two groups was 30.4 ± 4.1 and 29.6 ± 3.9 (P = 0.79 and P

Table 1 Prevalence of clinical and laboratory findings and smoking in the whole population

	Cases (138) yes/not	Controls (276) yes/not	RR (95% CI)	*P*
Smoking habit	81/57	108/168	1.5 (1.2-1.8)	0.000
Fasting glucose ⩾ 110 mg/dL	84/54	110/166	1.5 (1.25-1.9)	0.000
Abdominal obesity	98/40	107/169	1.8 (1.5-2.2)	< 0.0001
Hypertriglyceridemia	72/66	131/145	1.1 (0.8-1.3)	0.35
Low HDL-cholesterolemia	91/47	146/130	1.2 (1.1-1.5)	0.008
Hypertension	94/44	111/165	1.7 (1.4-2.0)	< 0.0001

RR: Risk ratio; CI: Confidence interval; HDL: High-density lipoprotein.

= 0.81, respectively). Sex distribution between the two groups did not show significant differences (χ^2, $P = 0.95$).

Among the 138 VTE patients, 112 (81%) had concomitant NAFLD, whereas 84 out of 276 (30%) controls suffered from NAFLD (RR = 2.7, 95% CI: 2.2-3.2, $P < 0.0001$). The RRs of smoking habit and single components of MS are shown in Table 1.

Factor V G1691A and/or prothrombin G20210A polymorphisms (major determinants) were not detected in 80 out of 138 cases (58%) and in 188 out of 276 controls (68.1%). A higher prevalence of NAFLD in patients with VTE but without inherited thrombophilia versus controls was also confirmed on the basis of this further selection; 62 patients in cases and 61 in controls (RR = 2.4, 95% CI: 1.9-3, $P < 0.0001$). When predicting NAFLD, VTE played an important role, which confirmed the aforementioned findings, but also smoking habit and some MS components gave a good prediction (Table 2). In contrast, age, BMI and sex did not enter the model, because their significance was > 0.1. The presence of VTE was well predicted by grade of steatosis, as revealed by US (OR = 1.9, 95% CI: 1.05-3.8, $P < 0.0001$).

DISCUSSION

Our main finding was a significantly higher prevalence of NAFLD in idiopathic VTE patients than in controls, which was confirmed after adjusting for inherited thrombophilia. Although these results were partially expected, they were highlighted for the first time in the present study. What is more, this report extends previous data[16] on MS. In a recent report[24], rather than considering "all-or-nothing" definitions for MS, the additive effect of having more than one of the MS features has been considered. This is a controversial point. However, what if physicians use an indirect parameter of MS presence, e.g. NAFLD? The present study supports a significant correlation between every single component of MS and VTE, even though the strictest association was demonstrated between NAFLD, a further expression of MS as a whole[22], and VTE, which by-passes the restrictive criteria of MS.

These data lend credence to the possibility that VTE is an early clinical event in a generalized vascular disease

Table 2 Prediction of nonalcoholic fatty liver disease

	OR (95% CI)	*P*
Venous thromboembolism	1.8 (1.2-2.7)	< 0.0001
Fasting glucose ⩾ 110 mg/dL	1.0 (1.0-1.02)	0.04
Abdominal obesity	1.7 (1.14-2.5)	0.0001
Hypertension	1.02 (1.0-1.04)	0.03
Smoking habit	1.6 (0.8-2.3)	0.0002

Method: Stepwise; variable entered if $P < 0.05$, and removed if $P > 0.1$. OR: Odds ratio; CI: Confidence interval.

that involves venous and arterial circulation. Our results support the need for further studies to evaluate the risk of subsequent cardiovascular events in VTE patients without MS, but with NAFLD.

In trying to establish the complex interaction between VTE and NAFLD, we stress that they share common mechanisms. First of all, we should pinpoint the role of PAI-1. In fact, abdominal fat, liver steatosis and serum triglycerides levels have been shown to be significant and independent determinants of PAI-1 plasma level in an unselected sample of male adults upon adjustment for age and therapy[25]. Additionally, the pro-angiogenic factor, vascular endothelial growth factor, which is generally thought to be the main factor that determines VTE in patients with cancer[26], plays a key role in NAFLD[27]. Recent evidence has substantiated that NAFLD is associated with elevated circulating levels of ICAM-1, which throws further light on inflammation-related liver damage[28]. Another intriguing link is represented by smoking, which is a plain risk factor for the development of VTE. Indeed, this relationship could be justified by the presence of NAFLD. In fact, Yuan *et al*[29] have provided novel evidence to demonstrate that tobacco smoke exposure can accelerate the development of experimental NAFLD.

The limitations of the present study are as follows. Our control group comprised individuals who were referred for signs or symptoms initially suggestive of VTE, and it may not adequately represent a general healthy population. However, the prevalence of MS in our control group was comparable to that reported in the general Italian population[5], which suggests that, with a differently selected control group, our findings could have been comparable to those reported in the previous study. Although patients with cancer were excluded from our study, some VTE patients might have had occult cancer at the time of investigation. The impact of occult cancer on the components of MS is unknown; however, its impact on the results of our analysis was likely to have been low. With regard to the definition of idiopathic VTE, we have defined it as VTE that occurs in patients without the most common known risk factors. Based on our definition, other risk factors might have been missed, but this is unlikely to have significantly influenced our results. Another potential point of criticism relates to the size of the study, which was not very large.

In conclusion, an eventual association between VTE and NAFLD should be always pursued.

COMMENTS

Background

Venous thromboembolism (VTE) with an annual incidence of 1-2 events/1000 people in the general population is considered to be an emerging health problem.

Research frontiers

Metabolic syndrome affects > 20% of the whole population, and increases the cardiovascular risk by a blood hypercoagulability-related mechanism.

Innovations and breakthroughs

This is believed to be the first evidence to show a strict link between idiopathic VTE and nonalcoholic fatty liver disease (NAFLD). Smoking could increase the risk of VTE by worsening NAFLD.

Applications

Patients suffering from MS should be warned against their increased risk of VTE.

Peer review

In this paper, research on the prevalence of NAFLD in idiopathic VTE is presented, which is an interesting topic.

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BRIEF ARTICLE

Extrahepatic portal vein thrombosis in children and adolescents: Influence of genetic thrombophilic disorders

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Abstract

AIM: To explore the prevalence of local and genetic thrombophilic disorders as risk factors for portal vein thrombosis (PVT) in our series, the largest ever published in pediatric literature.

METHODS: We conducted a case-control study enrolling 31 children with PVT and 26 age-matched controls. All were screened for thrombophilia, including genetic disorders, protein C, protein S and homocysteine deficiencies. All coagulation parameters were studied at least 3 mo after the diagnosis of portal vein obstruction.

RESULTS: In our study we showed that most pediatric patients with PVT have local prothrombotic risk factors, which are probably the most important factors leading to PVT. However, there is a clear association between the presence of prothrombotic disorders and PVT, suggesting that these increase the risk of thrombosis in patients with local factors such as perinatal umbilical vein catheterization or sepsis.

CONCLUSION: Patients with PVT should be screened for inherited prothrombotic disorders regardless of a history of an obvious local risk factor.

Key words: Portal vein thrombosis; Children; Thrombophilic disorders; Protein C; Protein S

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INTRODUCTION

Portal vein thrombosis (PVT) is a common cause of portal hypertension. To date, the pathogenesis of PVT in children still remains unexplained, yet it is the major cause of portal hypertension in children and adolescents.

Variceal bleeding due to PVT is a recognized cause of upper gastrointestinal bleeding in children in developing countries[1,2]. Hereditary thrombophilias that are known to predispose to venous thrombosis and PVT include certain mutations of the prothrombin (*PTHR*), factor V Leiden (*FVL*) or methylenetetrahydrofolate reductase (*MTHFR*) genes or deficiency of one of the natural anticoagulant proteins C and S[3-6].

Some neonatal events such as abdominal surgery, sepsis or umbilical vein catheterization (UVC) are typically identified in patients with PVT[7], with an incidence of thrombosis complicating UVC reported in the literature as high as 44%[8-10]. Moreover, other factors, such as dehydration, also have been suggested to play a part in PVT development[11].

Finally, despite all efforts, the cause of the blocked portal vein remains obscure in 50% to 90% of children[12]. Unlike in adults, studies of thrombophilic disorders in children are scant, and to date only a few studies have evaluated the prevalence of hereditary thrombophilic disorders in children and adolescents with PVT[1,2,13,14].

The aim of our study was to explore the prevalence of local and genetic thrombophilic disorders as risk factors in PVT in our series, the larger ever published.

MATERIALS AND METHODS

A 2-year prospective case-control study (December 2006 to December 2008) was carried out at Bambino Gesù Children's Hospital in Rome, Italy. The study was conducted according to the principles of the Declaration of Helsinki, informed consent was obtained, and the authors' institutional review board approved the study.

We enrolled two groups of subjects for the study. Group 1 included 31 (20 male) Caucasian patients with PVT; mean age, 7 yr 8 mo (range: 11 mo - 18 yr 2 mo). Group 2 comprised 26 children (15 male) free of liver disease and thrombotic events, age matched with group 1, who were inpatient in our hospital during the study. Upper endoscopy was performed in all patients of Group 1 and showed signs of portal hypertension but not always the presence of varices.

PVT was diagnosed by Doppler ultrasound scan or angiography [14 patients underwent both these procedures and we found a very high concordance (98%)]; both procedures were performed by the same radiologist. Normal liver function tests or no other sign of liver disease was an inclusion criteria, as well as the absence of histological abnormalities on liver biopsy examination when performed.

All patients were screened for thrombophilia including genetic disorders (*MTHFR C677T, FVL, PTHR G20210A*) protein C (PC), protein S (PS) and homocysteine. All coagulation parameters were studied at least 3 mo after the diagnosis of portal vein obstruction to avoid falsely low levels related to active thrombosis. Abnormal values of coagulation factors might be observed in patients with PVT due to impaired liver synthesis. Due to the lack of standards to define PC or PS deficiency in this setting, we used a ratio of these levels to prothrombin rate lower than 0.7 as a working definition for these deficits. This definition should exclude those patients with low PC and PS levels related to impaired liver synthesis, which would also affect prothrombin rate.

None of the patients were on anticoagulant or antiplatelet therapy at the time of the study. Detailed history was obtained with special emphasis on history of umbilical catheterization (50%), umbilical sepsis (6%), admission to neonatal intensive care unit (72%), severe gastroenteritis and dehydration (6%), history of thromboembolism in the patients and their family members (3%), and history of parental consanguinity (1%).

Genetic and specific analysis

Genomic DNA was isolated from white blood cells by standard procedures. A 222 bp fragment of the Factor V gene, a 165 bp fragment of the Factor Ⅱ gene and a fragment of the MTHFR gene are amplified from human genomic DNA using specific primers and the amplicon is detected by fluorescence using a specific pair of probes consisting of two different oligonucleotides that hybridize to an internal sequence of the amplified fragment during the annealing phase of a PCR cycle. The same specific probes are also used to determine the genotype by performing a melting curve analysis.

PC and PS activity was measured by coagulometric assay. PC activity was measured using a specific activator extracted from southern copperhead snake venom (*Agkistrodon c. contortrix;* STA protein C, Roche). PS activity was determined based on the principle of activated factor V inhibition (STA protein S, Roche). Protein activity was expressed as a percentage of a reference plasma pool.

Statistical analysis

Continuous variables were compared with unpaired *t*-test. Categorical variables were compared with the Fisher exact test. The association between the presence of an abnormality and PVT was assessed with odds ratios and their 95% confidence intervals (CI). All analyses were performed with SPSS version 15 (Chicago, IL, USA).

RESULTS

The characteristics of the patients and controls are summarized in Table 1. In patients, the first manifestation of portal vein thrombosis occurred at a mean age of 4 year 9 mo (range: 6 mo to 16 year 2 mo). This was upper gastrointestinal (GI) bleeding in 87% of patients followed by splenomegaly in 13%. Eighty-one percent of the patients had varices at presentation, while 74% had splenomegaly. Sixty-eight percent of the patients had a history of a local prothrombotic factor (neonatal sepsis or umbilical vein catheterization). Only 1 patient with a local prothrombotic factor was present in the controls.

Congenital thrombophilic disorders

FVL mutation was found in 2 (7%) patients and heterozygous *G20210A* mutation was found in 3 (10%), while

Table 1 Characteristics of the patients (mean ± SD)

	Controls	Patients
Age	6 yr 9 mo	7 yr 8 mo
Sex	15 males	20 males
INR (%)	1.05 ± 0.09	1.35 ± 0.21
ALT (UI/L)	31.15 ± 13.83	25.45 ± 14.71
AST (UI/L)	37.23 ± 11.86	31.42 ± 11.90
GGT (UI/L)	16.46 ± 16.89	17.68 ± 18.40
Albumin (g/dL)	4.16 ± 0.48	3.88 ± 0.37
Bil/tot (mg/dL)	0.78 ± 0.13	0.81 ± 0.27
Bil/dir (mg/dL)	0.12 ± 0.11	0.18 ± 0.14
ALP (UI/L)	404.09 ± 154.90	515.97 ± 180.44

INR: International normalized ratio; ALT: Aspartate aminotransferase; AST: Alanine aminotransferase; GGT: γ-glutamyl transpeptidase; Bil/tot: Bilirubin/total; Bil/dir: Bilirubin/direct; ALP: Alkaline phosphatase.

homozygosity for these two mutations was not found in any patient. No control patient had mutations in these two genes.

MTHFR-C667T mutation was found in 16 (68%) patients, and 4 (13%) were homozygous for this mutation. The corresponding figures in the control group were 6 (23%) and 0 patients, respectively (Table 2). Therefore, the odds ratio for having at least an allele with the *MTHFR-C677T* polymorphism in patients with portal vein thrombosis was 7.00 (95% CI: 2.15-22.85), suggesting that this polymorphism could increase the risk of PVT. However, intriguingly enough, levels of homocysteine in controls were similar to that found in cases ($P = 0.28$), and all subjects had normal values.

Coagulation inhibitor protein deficiency

Four patients presented PC levels compatible with prot C deficiency and 4 had PS levels compatible with prot S deficiency. We also investigated their parents to show any prot C alteration. Moreover, none of the controls had prot C deficiency while one had values consistent with prot S deficiency.

The overall frequency of inherited thrombophilic abnormalities (excluding mutations in *MTHFR*) in cases was 32% (8 patients had only one factor, one patient had two factors and one patient had three factors). This prevalence was significantly lower in controls (1/26, 4%) (Table 3). Thus, the OR of having any inherited prothrombotic disorder (excluding *MTHFR* polymorphism) in patients with portal vein thrombosis as compared to controls was 11.91 (95% CI: 1.41-100.77).

Eight patients (26%) had neither a thrombophilic nor a local factor (idiopathic portal vein thrombosis). This figure is in keeping with previously published series of adult portal vein thrombosis. Thirteen patients (42%) had only local factors, eight (26%) had both local and thrombophilic factors and only 2 patients (6%) had isolated inherited thrombophilic factors with no history of a local factor.

There were no associations between the presence of an inherited prothombotic disorder and the manner of initial presentation of PVT (splenomegaly or GI bleeding). However, patients with inherited thrombophilic factors were less likely to have varices at the time of presentation (6 out of 10 patients) as compared with patients without (19 out of 21; $P = 0.045$).

No recurrent thrombotic events were recorded in a 24 mo long follow-up, both in patients with and without prothrombotic disorders.

Table 2 Distribution of type and prevalence of mutations in study subjects *n* (%)

Type of mutations	Prevalence of mutations		
	Control (26)	Case (31)	Total (57)
Normal	20 (76.9)	10 (32.3)	30 (52.6)
Abnormal *MTHFRC677T*			
Heterozygous	6 (23.1)	17 (54.8)	23 (40.4)
Homozygous	0 (0)	4 (12.9)	4 (7.0)

$P = 0.001$. *MTHFR*: Methylenetetrahydrofolate reductase.

Table 3 Frequency of inherited thrombophilic abnormalities *n* (%)

Any thrombophilic factor	Control	Case	Total
No	25 (96.2)	21 (67.7)	46 (80.7)
Yes	1 (3.8)	10 (32.3)	11 (19.3)
Total	26 (100)	31 (100)	57 (100)

$P = 0.008$ (Fisher's exact test).

DISCUSSION

In this study we show that most patients with pediatric portal vein thrombosis have a history of a local prothrombotic factor, such as sepsis or umbilical vein catheterization, a figure much higher than that reported for adult portal vein thrombosis (around 30%)[15]. Therefore, and distinctly from adult PVT, local factors seem clearly to be the major players implicated in the development of PVT in children. However, a major finding of this study is that inherited disorders of coagulation are also frequently found in these patients (38%, as compared with 4% in controls), though most times in association with a local factor. This suggests, on one hand, that inherited thrombophilic disorders might facilitate the development of PVT thrombosis after a "local" event. On the other hand, since the presence of a thrombophilic disorder might have an impact on the management and follow-up of these patients, our data support the notion that children with PVT should be thoroughly investigated for the presence of a thrombophilic factor, even if an obvious history of a local factor is present.

The most frequent thrombophilic disorder was a deficit in naturally occurring anticoagulants (proteins C or S). It is possible, however, that the prevalence of coagulation inhibitor protein deficiency might be overestimated, since these factors might decrease due to altered liver synthesis and decreased hepatic blood flow secondary to the thrombosis, as already suggested in literature[6,16]. Notably, some

Table 4 Frequency of thrombophilic disorders in children and adolescents with portal vein thrombosis *n* (%)

Study	Coagulation inhibitor protein deficit		Gene mutations		
	PC	PS	*FVL*	*PTHR*	*MTHFR*
Dubuisson *et al*[13] (*n* = 20)	9 (45)	13 (65)	NP	NP	NP
Uttenreuther-Fischer *et al*[2] (*n* = 23)	NP	NP	2 (9)	NP	NP
Heller *et al*[1] (*n* = 24)	NP	NP	4 (17)	0	1 (4)
Pinto *et al*[12] (*n* = 14)	6 (43)	3 (21)	0	1 (7)	3 (21)
Current study (*n* = 31)	4 (13)	4 (13)	2 (6.5)	3 (9.7)	16 (67.7)

PC: Protein C; PS: Protein S; *FVL*: Factor V Leiden; *PTHR*: Prothrombin; *MTHFR*: Methylenetetrahydrofolate reductase; NP: Not provided.

studies have already shown a rise in the concentration of coagulation inhibitor proteins after a surgical correction directly bypassing the venous obstruction[17,18], confirming this hypothesis. However, even with our restrictive working definition of prot C and S deficit (less than 0.7 × prothrombin rate), we have found a high prevalence of these disorders, suggesting that their role is more relevant in pediatric than in adult PVT. Larger numbers with detailed family history (difficult to recruit in this setting) would be required to gain further insight into this finding.

In our population the *MTHFR-C677T* polymorphism was much more frequent in patients than in controls, suggesting that it behaves as a risk factor for PVT. However, in our cohort no difference in the levels of homocysteine between controls and patients was found. Thus, this polymorphism is not always associated with high plasma levels of homocysteine[19,20], even in patients with documented thrombotic events and no other risk factor for thrombophilia. This raises the question of whether the *MTHFR* gene polymorphism, without hyperhomocysteinemia, may itself contribute to thrombophilia. On the other hand, intermittent hyperhomocysteinemia may occur, which is not easily detectable even if clinically significant. In addition, the interpretation of homocysteine levels in these patients is problematic. Dietary imbalances, such as an inadequate intake of folate and vitamin B12 which are needed to break down excess homocysteine or methionine overabundance from dietary protein, may play a critical role in homocysteine metabolism[21,22].

Although the association between the *MTHFR* mutation and thrombosis has not yet been fully clarified[6,23,24], anticoagulation may be indicated in patients with *MTHFR* mutation (either homozygous or heterozygous, with or without hyperhomocysteinemia)[25] with previous thrombotic events and other thrombotic risk factors (pregnancy, oral contraceptives, surgery, sepsis and immobilization). Therefore, our data suggest that the presence of *MTHFR* mutations should be investigated in all pediatric patients with PVT. In addition, if hyperhomocysteinemia is present, therapy with folic acid and B6 and B12 vitamins should be instituted.

Another finding of this study was the lower prevalence of GI varices in patients with prothrombotic disorders. This might be an association by chance, or could reflect that those patients with a thrombophilic factor were more likely to be diagnosed in a phase of "recent thrombosis", when varices still have not developed. At any rate, no differences were observed in the clinical evolution of PVT between patients with genetic anomaly and those without.

In summary, most pediatric patients with PVT have local prothrombotic factors, which are probably the most important factors leading to PVT (Table 4). However, there is a clear association between the presence of prothrombotic disorders and PVT, suggesting that these increase the risk of thrombosis in patients with local factors such as perinatal UVC or sepsis. Patients with PVT should be screened for inherited prothrombotic disorders regardless of a history of an obvious local risk factor. Future trials should evaluate the role of prophylactic low molecular weight heparins in children requiring UVC, especially in those with a family history of thrombotic events or other thrombotic risk factors.

COMMENTS

Background

Portal vein thrombosis (PVT) is a common cause of portal hypertension. To date, the pathogenesis of PVT in children still remains unexplained despite the fact that it is the major cause of portal hypertension in children and adolescents. Unlike in adults, studies of thrombophilic disorders in children are scant, and to date only a few studies have evaluated the prevalence of hereditary thrombophilic disorders in children and adolescents with PVT.

Research frontiers

To date, many pediatric patients with PVT have local prothrombotic factors, which are probably the most important factors leading to PVT. However, there is a clear association between the presence of prothrombotic disorders and PVT, suggesting that these increase the risk of thrombosis in patients with local factors such as perinatal umbilical vein catheterization (UVC) or sepsis. Patients with PVT should be screened for inherited prothrombotic disorders regardless of a history of an obvious local risk factor. Future studies should evaluate the role of prophylactic low molecular weight heparins in children requiring UVC.

Innovations and breakthroughs

This series is the larger ever published so far. In this study the authors show that most patients with pediatric PVT have a history of a local prothrombotic factor, a figure much higher than that reported for adult portal vein thrombosis (around 30%). The authors suggest extending the thrombophilic screening of three different genetic mutations to better analyze this population.

Applications

Patients with PVT should be screened for inherited prothrombotic disorders regardless of a history of an obvious local risk factor.

Peer review

This is a well written and important contribution to the pediatric literature. The paper describes the wider case series of patients with these conditions and few published series are present at the moment, the data could be of interest for the readers.

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BRIEF ARTICLE

Tissue factor in predicted severe acute pancreatitis

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Abstract

AIM: To study tissue factor (TF) in acute pancreatitis and evaluate the role of TF as a predictive marker of severity.

METHODS: Forty-nine consecutive patients admitted to Lund University Hospital, fulfilling the criteria of predicted severe acute pancreatitis (AP), were recruited prospectively between 2002 and 2004. Blood samples for TF analyses were drawn at inclusion in the study and 12 h, 1 d and 3 d later.

RESULTS: Twenty-seven patients developed mild AP, and 22 patients severe AP. At inclusion in the study, the groups were comparable with respect to gender, aetiology, Acute Physiology and Chronic Health Evaluation Ⅱ score, and duration of pain. At inclusion in the study and at 12 h, TF was higher in the severe AP group (P = 0.035 and P = 0.049, respectively). After 1 and 3 d, no differences in TF levels were noted. Interleukin (IL)-6 was significantly higher in the severe AP group at all of the studied time points. C-reactive protein (CRP) was significantly higher in the AP group at 1 and 3 d. In receiver operating characteristic-curves, the area under the curve (AUC) for TF was 0.679 (P = 0.035) at inclusion in the study, and a cut off level for TF of 40 pg/mL showed a sensitivity of 71% and a specificity of 67%, whereas corresponding AUC for IL-6 was 0.775, P = 0.001, and for CRP was 0.653. IL-6 showed better AUC-values than TF at all time points studied.

CONCLUSION: TF-levels are raised early in severe AP. TF as an early predictive marker of severe AP is superior to CRP, but inferior to IL-6.

Key words: Acute pancreatitis; Coagulation; Prediction of severity; Tissue factor

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INTRODUCTION

Severe acute pancreatitis (AP) is one example of critical illness where both the inflammatory system and the coagulation system are to be considered as ticking bombs, where the most extreme scenarios result in multiple organ dysfunction and disseminated intravascular coagulation. Microcirculatory disturbances with micro vascular thromboses appear to play an important role both in the inflamed pancreas itself and in remote organ failure[1,2]. Clinical evidence is still sparse[3-5], but several experimental studies have suggested an important role of the coagulation system in the pathophysiology of AP[6-8]. One key to the cross-talk

between inflammation and coagulation are proteases, with enzymatic capacity to activate both inflammation and coagulation. Coagulation factors, such as factor Ⅶ (FⅦ) and tissue factor (TF), as well as thrombin, can bind to protease activated receptors (PARs) on various cells and elicit intracellular signalling, resulting in modulation of inflammatory response[9]. The PAR family has at least four members (PAR 1-4) where TF-FⅦ has been shown to be able to act through PAR-2, while TF-FⅦ-FX also activates PAR-1. PAR-2 is the only PAR not activated by thrombin[10].

Tissue factor is a trans-membrane glycoprotein, initiating the most important pathway of coagulation[11,12]. Tissue factor is expressed in the vascular adventitia, but may also be expressed in micro-particles which can be shed from leukocytes, endothelial cells, vascular smooth muscle cells and platelets[13]. In the normal setting TF is not in contact with circulating blood. When vessels are injured or when TF-expressing cells are stimulated by circulating pro-inflammatory cytokines or lipopolysaccharide (LPS), TF is exposed to the bloodstream. TF then binds and activates factor Ⅶ. Factor Ⅶ is a vitamin K-dependent trypsin-like serine protease, produced in the liver. It circulates in an inactive form, and requires the action of its allosteric regulator, TF, to convert it to the active enzyme (FⅦa). The TF-factor Ⅶ complex initiates coagulation by activating FX, eventually resulting in conversion of pro-thrombin to thrombin. Thrombin cleaves fibrinogen, resulting in abundant fibrin production and the formation of a clot. The activity of TF is counterbalanced by circulating tissue factor pathway inhibitor (TFPI). In addition to its well-established role in coagulation, TF, and to a lesser extent FⅦ, have also been associated with various other physiological processes of gene transcription, apoptosis and cytoskeleton reformation, such as in inflammation, sepsis, metastasis, angiogenesis and atherosclerosis, where the TF-FⅦa complex acts as a signalling receptor[14-17]. The role of TF/FⅦa signalling in inflammatory conditions is confirmed by TF/FⅦa regulated expression of the pro-inflammatory cytokine interleukin (IL)-8 in keratinocytes[18], and a role in the regulation of both IL-6 and IL-8 expression in monocytes/macrophages[19]. Confirming the effect of FⅦa on expression of interleukins, recombinant FⅦa administered to healthy humans caused a three- to four-fold increase in plasma levels of IL-6 and IL-8[20]. A role of TF/FⅦa signalling in the regulation of inflammatory genes has been demonstrated in LPS-stimulated macrophages, where TF-FⅦa signalling activated genes coding for tumor necrosis factor-α, IL-6, and IL-8[21].

Recent clinical studies have suggested a potential role of coagulation variables, such as TF, TFPI and D-dimer, in predicting risk of developing organ failure and severe AP[22-24]. However, the evidence supporting their use as predictors of severity of AP is still weak, compared to C-reactive protein (CRP) and IL-6, which to date are the most well-documented laboratory parameters to predict severe AP[25-28].

The present study aimed to investigate plasma levels of TF in the initial phase of predicted severe AP, and to assess the ability of this biochemical marker to predict severe AP.

MATERIALS AND METHODS

Consecutive patients admitted to Lund University Hospital with the clinical diagnosis of acute pancreatitis, were recruited prospectively between June 2002 and December 2004. Inclusion and exclusion criteria are listed in Table 1.

Written informed consent was obtained and the study was approved by the local research ethics committee. This study was part of a prospective single-centre study on early enteral nutrition *vs* total parenteral nutrition in AP, where parts of the data on IL-6 and CRP have been published[29]. Venous blood was taken for measurement of plasma levels of TF, FⅦ, fibrinogen, IL-6 and CRP. Not all markers were measured at all time points in the study. TF and IL-6 were measured at inclusion, after 12 h, and after 1 and 3 d. CRP was measured at inclusion, and after 1 and 3 d. Fibrinogen and FⅦ were only measured at inclusion in the study.

Descriptive data were recorded including age, gender, aetiology, time from onset of pain to inclusion in the study, Acute Physiology and Chronic Health Evaluation (APACHE) Ⅱ score on day 1 and 3, organ failure, and mortality. The severity of pancreatitis was assessed according to the Atlanta classification[30].

Blood samples and assays

Peripheral blood samples were taken from each patient on study inclusion, at 12 h, and after 1 and 3 d. Admission plasma levels of FⅦ were analysed, and to detect the prevalence of fibrinolysis and fibrinogen consumption at admission, plasma fibrinogen was analysed. Fibrinogen is an acute phase protein, affected by pathologic proteolysis such as in disseminated intravascular coagulation, where low levels of fibrinogen are to be expected. TF, IL-6 and CRP were analysed at repeated time points during three days after inclusion in the study.

Tissue factor and fibrinogen were collected using citrate tubes, and ethylenediaminetetraacetic acid tubes were used for IL-6 and CRP. All samples were centrifuged at 2200 *g* for 10 min (3200 r/min, rotor diameter 19.1 cm). The plasma was decanted and stored at -70℃ until further analysis.

TF and FⅦ were assessed by enzyme-linked immunosorbent assay (ELISA)-kits according to the manufacturer's instructions (Assaypro St. Charles, MO, USA). The TF-ELISA recognizes TF-apo, TF and TF-Ⅶ complexes. The FⅦ-ELISA detects free FⅦ and FⅦa, as well as complexes with TF, TF/factor Ⅶ and TF/FⅦa.

Fibrinogen was analysed by Sysmex CA-7000 (Sysmex Corporation, Kobe, Japan) according to the operator's manual. The procedure involves mixing citrate plasma with buffer. After incubation, coagulation was initiated by adding an excess of thrombin. The time between addition of thrombin and coagulation was registered photo-optically and is inversely proportional to the concentration of fibrinogen.

IL-6 was measured by an ELISA-kit according to the manufacturer's instructions (Quantikine, R6D systems Europe, Abingdon, UK). CRP was measured by Cobas

Table 1 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
> 18 yr	Acute pancreatitis due to surgery
Abdominal pain	Trauma
Amylase > 3 times upper normal limit	Cancer
Onset of abdominal pain within 48 h	Inflammatory bowel disease
APACHE Ⅱ score > 8 and/or	Stoma
CRP > 150 and/or	Short bowel
Peripancreatic fluid collection on CT	Chronic pancreatitis

APACHE: Acute Physiology and Chronic Health Evaluation; CRP: C-reactive protein; CT: Computed tomography.

6000 (Roche Corporation, Basel, Switzerland) according to the operator's manual. The complex binding between CRP and CRP monoclonal antibodies attached to latex particles was registered as an increase in absorbance, measured photo-optically, and the increase in absorbance was related to the concentration of CRP.

Statistical methods

Data are presented as median and interquartile range, when applicable. Outliers are not shown in the box-plots, but are included in all calculations. Comparisons between groups were performed with the χ^2 test for binary data or Fisher's exact test for small samples. Continuous variables were compared with the Mann-Whitney *U*-test. To evaluate TF as a predictor of severe AP, receiver operating characteristics (ROC) curves were plotted and positive likelihood ratios (PLR) and negative likelihood ratios (NLR) were calculated to detect optimal cut-off levels. As a comparison, figures calculated from levels of CRP and IL-6 were used, as they are known to be good predictors of severity, IL-6 already at admission[27] while CRP peaks about 48 h later.

In a ROC curve, the true positive rate (sensitivity) is plotted in function of the false positive rate (100 - specificity) for different cut-off points. Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold. A test with perfect discrimination (no overlap in the two distributions) has a ROC plot that passes through the upper left corner (100% sensitivity, 100% specificity). The closer the ROC plot is to the upper left corner, and the greater the area under the curve, the higher the overall accuracy of the test is[31]. The Likelihood Ratio (LR) is the likelihood that a given test result would be expected in a patient with the target disorder, compared to the likelihood that the same result would be expected in a patient without the target disorder.

Statistical analyses were performed with SPSS version PASW Statistics 18 (SPSS Inc, Chicago, IL, USA).

RESULTS

Patient characteristics

According to the Atlanta classification, 22 patients (45%) fulfilled the criteria of severe AP, and 27 patients (55%) were classified as having mild AP. One patient in the severe AP group died, rendering an overall mortality rate of 2.0%. At inclusion in the study, the groups with mild and severe pancreatitis were comparable with respect to gender, aetiology, APACHE Ⅱ score, and duration of pain prior to inclusion. Age was lower in the severe AP group, compared to the mild AP group. Patient characteristics and laboratory variables at time of inclusion are presented in Table 2.

Table 2 Patient characteristics and laboratory variables at time of inclusion

	MAP (n = 27)	SAP (n = 22)	P
Age (yr)[1]	76 (63-85)	63 (56-77)	0.042
Sex (M:F)	14:13	10:12	0.664
APACHE Ⅱ[1]	9 (8-11)	9 (7-13)	0.860
Aetiology			
Biliary	15	16	0.248
Alcohol	4	3	1.000
ERCP	3	1	0.617
Unknown	5	2	0.436
Duration of pain (h)[1]	34 (21-43)	25 (22-29)	0.160
Amylase[1]	8.2 (2.7-13.7)	9.8 (4.3-15.3)	0.690
IL-6 (pg/mL)[1]	100 (55-210)	275 (158-315)	0.001
CRP (mg/mL)[1]	106 (69-167)	173 (104-209)	0.071
Tissue factor (pg/mL)[1]	35 (23-50)	49 (36-101)	0.035
Fibrinogen (g/L)[1]	4.8 (4.4-6.2)	4.0 (3.8-7.2)	0.047
FⅦ (ng/mL)[1]	155 (46-294)	136 (88-296)	0.608

[1]Values are expressed as median and inter-quartile range. MAP: Mild acute pancreatitis; SAP: Severe acute pancreatitis; APACHE: Acute Physiology and Chronic Health Evaluation; ERCP: Endoscopic retrograde cholangiopancreatography; IL-6: Interleukin-6; CRP: C-reactive protein; FⅦ: Factor Ⅶ.

Markers

Because some blood samples were not taken properly, there are different numbers of patients at the different time points. At inclusion in the study, TF was higher in the severe AP group, whereas fibrinogen was lower in the severe AP group compared to the group with mild AP [Figure 1, tissue factor (pg/mL)].

There was no difference in FⅦ-levels between the groups (P = 0.608). A large variation in inter-individual levels of FⅦ was noted [Figure 2, scattergram of FⅦ plasma levels at admission (ng/mL)]. IL-6 was higher in the severe AP group (P = 0.001), and CRP showed a tendency towards higher levels in the severe AP group (P = 0.071) at time of inclusion in the study (Table 2).

When looking at changes over time, TF was slightly higher in the severe AP group at 12 h (P = 0.049). After 1 and 3 d no differences in TF levels were noted between the mild and the severe AP group [Figure 1, tissue factor (pg/mL)].

IL-6 peaked at 12 h and was significantly higher in the severe AP group at all of the studied time points (at inclusion P = 0.001, 12 h P < 0.001, 1 d P < 0.001 and 3 d P = 0.000, respectively). CRP peaked at day 3, and was significantly higher in the AP group at 1 and 3 d (P = 0.001 and P < 0.001, respectively).

Prediction of severity

To evaluate the utility of TF as an early predictor of se-

Table 3 Area under the curve-values, possible cut-off levels, sensitivity, specificity, positive and negative likelihood ratio for tissue factor

Time point[1]	AUC	*P*	Cut-off TF (pg/mL)	Sensitivity	Specificity	PLR	NLR
0	0.679	0.035	32	86	48	1.65	0.30
			40	71	67	2.14	0.43
			46	62	74	2.39	0.51
0.5	0.681	0.049	33	90	43	1.57	0.26
			41	78	56	1.79	0.39
			47	72	70	2.37	0.40
1	0.652	0.078					
3	0.621	0.151					

[1]Time points: 0 = inclusion in study, 0.5 = 12 h, 1 = 24 h, 3 = 3 d. AUC: Area under the curve; TF: Tissue factor; PLR: Positive likelihood ratios; NLR: Negative likelihood ratios.

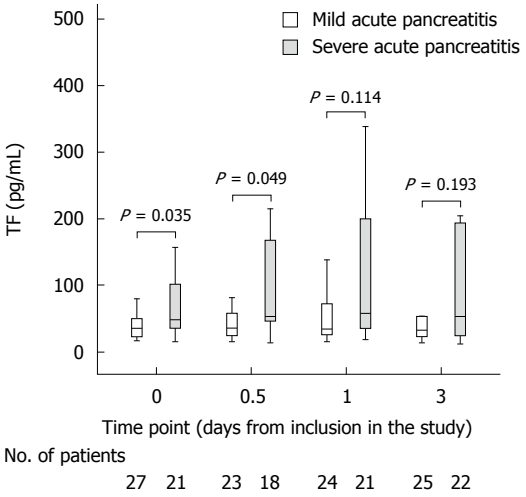

Figure 1 Tissue factor. Time points: 0 = inclusion in study, 0.5 = 12 h, 1 = 24 h, 3 = 3 d. TF: Tissue factor.

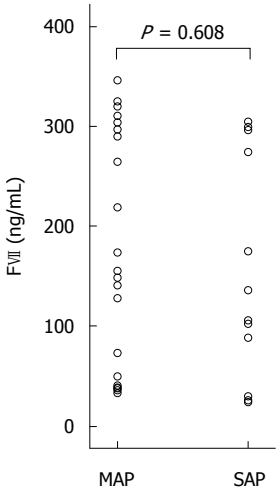

Figure 2 Scattergram of factor Ⅶ plasma levels at inclusion (ng/mL). FⅦ: Factor Ⅶ; MAP: Mild acute pancreatitis; SAP: Severe acute pancreatitis.

vere AP, ROC-curves were plotted for the time of inclusion (Figure 3A, ROC curves of TF, IL-6 and CRP at time of inclusion in the study), and for 12 h (Figure 3B, ROC curve of TF and IL-6 at 12 h after inclusion in the study), 1 d (results not shown) and 3 d after inclusion in the study (Figure 3C, ROC curves of TF, CRP and IL-6 at 3 d after inclusion). As a comparison, ROC-curves were plotted for CRP and IL-6. Area under the curve (AUC) values at the different time points were studied. Based on these results, possible cut-off levels for TF are suggested at inclusion and after 12 h, based on sensitivity, specificity, PLR and NLR. Table 3 shows AUC-values, P-values, possible cut-off levels, sensitivity, specificity, PLR and NLR for TF (pg/mL).

DISCUSSION

Several previous studies on coagulation factors in AP have been published. In a study on 36 patients with AP, elevated levels of TF were detected at admission. In that study only 5 patients were classified as having moderate AP, while 31 had severe AP according to the Japanese Severity Score[22]. A correlation between higher levels of TF and development of organ failure was demonstrated, but in contrast to the results from the present study no correlation with overall severity was detected. In the present study, TF was higher in severe AP compared to mild AP at inclusion in the study, i.e. close to admission, and after 12 h.

The levels of fibrinogen in both mild and severe AP were in the higher span or above the reference for normal human plasma levels, consistent with fibrinogen being an acute phase protein. A slightly lower level of fibrinogen was noted in the group with severe AP at inclusion in the study. The results are, however, hard to interpret as fibrinogen is an acute phase protein and the level of fibrinogen in the severe AP group was just above normal level. It should be stressed that the result for fibrinogen was of weak significance, and further studies of other parameters of fibrinolysis, such as D-dimer and fibrin degradation products should be conducted in order to tell whether early fibrinolysis is the explanation for the lower levels of fibrinogen in the severe AP group.

In a study on 91 patients with AP, D-dimer, prothrombin time and fibrinogen were different when comparing patients developing organ failure and patients not developing organ failure, both at admission and 24 h later. D-dimer was the best predictive marker of organ failure (sensitivity 90%, specificity 89%)[23]. In a study on 139 patients with AP, the levels of antithrombin Ⅲ (AT-Ⅲ),

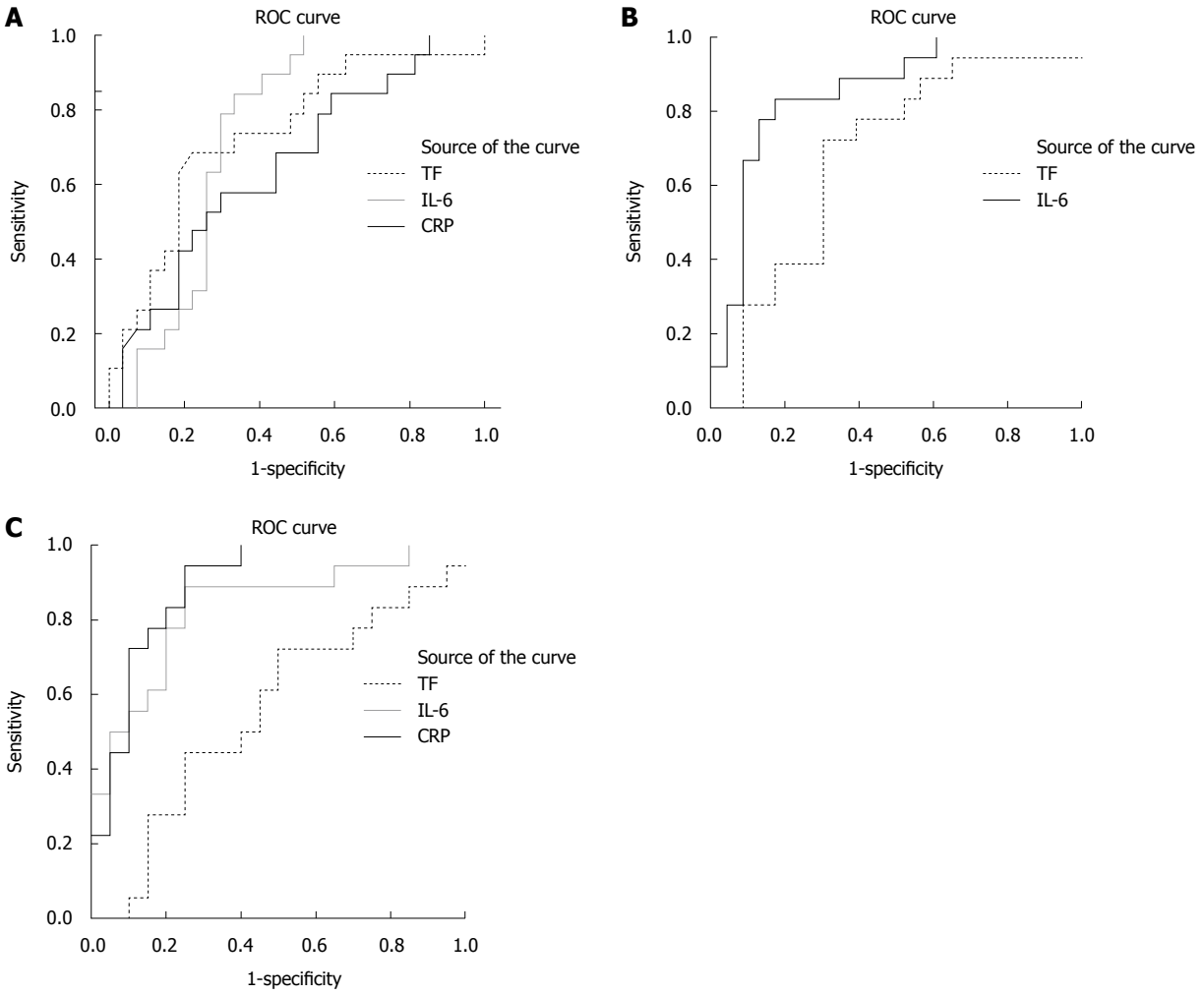

Figure 3 Receiver operating characteristic curves. A: Receiver operating characteristic (ROC) curves of tissue factor (TF), interleukin-6 (IL-6) and C-reactive protein (CRP) at time of inclusion in the study; B: ROC curves of TF and IL-6 at 12 h after inclusion in the study; C: ROC curves of TF, IL-6 and CRP at 3 d after inclusion.

fibrin/fibrinogen degradation products, platelet count, D-dimer, and antithrombin-AT-Ⅲ complex at admission were associated with severity and prognosis of AP. AT-Ⅲ, fibrin/fibrinogen degradation products, platelet count, D-dimer, and thrombin-AT-Ⅲ complex at admission showed better area under the ROC curve values compared to CRP. AT-Ⅲ was the best predictor of fatal outcome (sensitivity 81%, specificity 86%)[32].

In experimental studies, deficiency of FⅦ has been shown to reduce inflammation[33,34] and high levels of FⅦ have been suggested to be associated with ischemic heart disease and inflammation[35,36]. In the present study, concentrations of FⅦ did not differ between the mild AP group and the severe AP group, and hence levels of FⅦ do not seem to be affected in the early course of the disease. The large variation in levels of FⅦ is consistent with reported findings on a strong contribution of the FⅦ genotype to levels of FⅦ. Different FⅦ genotypes can result in up to several-fold differences in mean FⅦ levels[37].

Early recognition of patients at risk of developing severe AP with multiple organ failure and high risk of mortality remains a challenge, despite the use of multifactor scoring systems such as APACHE-Ⅱ and Ranson's score[38]. Obesity, age, alcohol consumption and use of tobacco are known to predispose to a severe disease course[39,40]. The most widely used laboratory parameter to predict severity of AP and development of complications is CRP. A meta-analysis on the ability of IL-6 to predict severe AP concludes that these cytokines perform at an acceptable level in predicting severe AP[26]. The pooled IL-6 sensitivities ranged between 81.0% and 83.6% and specificities between 75.6% and 85.3% with PLRs of 3.43, 4.90 and 4.40 for days 1, 2 and 3, respectively. The IL-6 AUCs were 0.75, 0.88 and 0.85 for days 1, 2 and 3, which are in accordance with the AUCs for IL-6 in the present study.

Data concerning the role of coagulation variables as predictors of severe AP are scarce. In a study of 44 patients with AP, TFPI measured at admission was shown to be related to severity[24]. Among the three variables in the present study, fibrinogen, FⅦ and TF, TF was significantly raised at admission, when comparing the severe and the mild AP group. With this result in mind, TF was explored as a marker of severity at four different time points, by area under ROC-curves. At admission and after 12 h, the AUC for TF was 0.68, and when evaluating different cut-off points the best PLR was 2.4, with a sensitivity of 62%

and a specificity of 72%, which implies a quite low impact on the likelihood of severe disease, much less impact than IL-6 at corresponding time points.

We conclude that levels of TF, but not FVII, are higher in "true severe" AP than in those patients with predicted severe AP who turn out to develop mild AP. Our results stress the need of more reliable predictors of severity, as only 45% of the patients in our study with predicted severe disease actually developed severe AP. The value of TF as a predictive marker of severe AP early in the course of the disease is not as good as IL-6, but superior to CRP. The results do not indicate a role for TF as a valuable predictive marker of severity on its own. The higher levels of TF in the early course of severe AP suggest, however, a potential role of TF in the development of severe disease, and may reflect a window for therapeutic inhibition of TF in AP.

COMMENTS

Background

Acute pancreatitis affects about 20-40/100 000 inhabitants each year. One fifth of these patients will develop a severe form of AP with multiple organ failure and a high risk of death. There is no reliable marker to early predict which patients will develop the severe form. In severe disease, such as AP, a close interplay between coagulation and inflammation is known to exist, and take part in the development of the disease. In this paper, tissue factor, which is a key player in the crosstalk between inflammation and coagulation, is measured in the plasma of patients with predicted severe pancreatitis.

Research frontiers

Data concerning the role of coagulation variables as predictors of severe acute pancreatitis (AP) are still sparse. The results from one study on patients with AP, showed that levels of tissue factor (TF) were related to the development of pancreatic necrosis in alcoholic severe AP, but no association with overall severity was demonstrated (Sawa *et al* 2006). In another study of AP, the coagulation parameters D-dimer, pro-thrombin time and fibrinogen were different in the group of AP patients developing organ failure compared to the patients who did not develop organ failure, both at admission and 24 h later. D-dimer was the best predictive marker of organ failure (Radenkovic *et al* 2009). In yet another study on AP, the levels of the coagulation parameters antithrombin Ⅲ (AT-Ⅲ), fibrin/fibrinogen degradation products, platelet count, D-dimer, and thrombin-AT-Ⅲ complex at admission were associated with severity and prognosis of AP. AT-Ⅲ was the best predictor of fatal outcome (Maeda *et al* 2006).

Innovations and breakthroughs

The authors show that levels of TF measured early in the course of the disease are higher in patients who develop severe AP. These results are consistent with the possible role of coagulation variables in the development of AP.

Applications

The role of TF as an early predictor of severe AP is inferior to interleukin-6, which has been shown to be of value in various previous studies, however, TF is superior to the most frequently used laboratory parameter, C-reactive protein. The results indicate a role for TF in the development of severe AP, and the effect of tissue factor pathway-inhibitors in AP should be studied.

Terminology

Acute pancreatitis is an acute inflammation of the pancreatic gland, most often elicited by alcohol ingestion or gall stone disease. Tissue factor is located in the membrane of various cells surrounding the blood vessels throughout the body, and is exposed to circulating blood when vessels are ruptured or may be expressed by white blood cells or cells on the inside of blood vessels in inflammatory conditions, such as acute pancreatitis. When tissue factor binds to factor Ⅶ, circulating in the blood, the coagulation cascade is initiated, but tissue factor - factor Ⅶ may also modulate the inflammatory response.

Peer review

This clinically relevant study of the predictors of pancreatitis severity looks fine.

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BRIEF ARTICLE

Role of serotonin in development of esophageal and gastric fundal varices

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Abstract

AIM: To determine the effect of free serotonin concentrations in plasma on development of esophageal and gastric fundal varices.

METHODS: This prospective study included 33 patients with liver cirrhosis and 24 healthy controls. Ultrasonography and measurement of serotonin concentration in plasma were carried out in both groups of subjects. The upper fiber panendoscopy was performed only in patients with liver cirrhosis.

RESULTS: The mean plasma free serotonin levels were much higher in liver cirrhosis patients than in healthy controls (219.0 ± 24.2 nmol/L *vs* 65.4 ± 18.7 nmol/L, $P < 0.0001$). There was no significant correlation between serotonin concentration in plasma and the size of the esophageal varices according to Spearman coefficient of correlation ($r_s = -0.217$, $P > 0.05$). However, the correlation of plasma serotonin concentration and gastric fundal varices was highly significant ($r_s = -0.601$, $P < 0.01$).

CONCLUSION: Free serotonin is significant in pathogenesis of portal hypertension especially in development of fundal varices, indicating the clinical value of serotonergic receptor blockers in these patients.

Key words: Serotonin; Portal hypertension; Esophageal varices; Fundal varices; Platelets

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INTRODUCTION

Portal hypertension has been increasingly regarded as a multi-organ disease with complex blood flow changes in the systemic and splanchnic vascular network.

The hepatic stellate cell (HSC) has a significant position in the sinusoid for regulation of portal flow. During liver damage, HSCs are "activated" which leads to HSC transformation into myofibroblast-like cells with a resulting increased collagen production[1,2]. Several mitogens are included in the triggering of HSC proliferation: platelet-derived growth factor (PDGF), insulin-like growth factor 1 and connective-tissue growth factor. During activation,

HSCs acquire the ability to express PDGF receptors on the cell membrane surface[3]. The HSC membrane contains numerous receptors whose expression is increased with the extent of liver damage, to which different vasoconstrictors are bound: catecholamines, endothelin, angiotensin I and Ⅱ, leukotrienes and serotonin [5-hydroxytriptamin (5-HT)][4,5].

Serotonin, at the level of hepatic sinusoids, causes endothelial fenestrae contractions of liver sinusoids through 5-HT_1 receptors mediated by a Ca^{2+}-dependent process. Due to different proinflammatory mediators releasing from the damaged liver, it comes to platelet adherence to sinusoidal endothelium, translocation into Disse's space and serotonin release. Thereafter, serotonin binds to receptors (5-HT_{2A}, 5-HT_{1B}, 5-HT_{1F} and 5-HT_7) which are expressed on HSC and hepatocytes, which additionally interferes with HSC proliferation[6,7].

The aim of our study was to determine to what extent a free serotonin concentration in plasma has an effect on development of esophageal and gastric fundal varices.

MATERIALS AND METHODS

The study included 33 patients with liver cirrhosis who were examined and treated at the Clinic of Gastroenterology, Clinical Center of Serbia, and 24 healthy subjects who made up the control group. The study was prospective and conducted during the period 2008-2009.

Ultrasonography was carried out by Toshiba Core Vision, with 3.5 MHz duplex Doppler convex tube in a standard procedure. Ultrasonography examined the liver size, echo structure of the hepatic parenchyma and possible focal changes with a view to rule out the patients with primary and secondary liver tumors from the study. To determine the spleen size, standard parameters were used, according to which in physiological conditions the spleen diameter measured in the X intercostal space exceeded no more than 12.0 cm and anteroposterior diameter was not over 5.0 cm.

The upper endoscopy was performed by endo-video system Olympus GIF-Q 165. To measure the esophageal varices size, Paquet's classification was used: I degree-lesser snake-like mucosal protrusions, Ⅱ degree-varices were predominating up to a half of the esophageal lumen radius, Ⅲ degree-varices were in contact at some points, and Ⅳ degree-heralds of the imminent rupture (cherry red spots)[8]. Endoscopic examination showed portal hypertensive gastropathy (snake skin mucosa) and varices of the gastric fundus.

Platelet poor plasma (PPP) was obtained from the venous blood which was collected in 3 mL original Vacutainer "BD" tubes, with 75 g/L K_3EDTA 0.072 mL. Blood samples were taken between 8 and 9 a.m. Platelet rich plasma (PRP) was obtained by low speed centrifugation (200 *g*, 10 min) on "Heraeus Digifuga GL". Exactly 1 mL of PRP was centrifuged at 1000 *g*, 10 min. The obtained PPP was separated and stored at -20℃ for no longer than 20 d[9].

The number of PPP serotonin samples was estimated in one series. One hundred μL plasma samples were spiked with 10 μL of original N-methyl serotonin solution (Recipe, Munchen), which was an internal standard. After that, PPP samples were deproteinized with 100 μL original deproteinating reagent (Recipe, München), and centrifuged at 10 000 *g*[10]. The obtained 20 μL supernatants were analyzed on reverse phase HPLC column (Recipe, München), with original mobile phase for serotonin (Recipe, München). Original "Recipe" external standard solution was used for calibration. The HPLC system consisted of "Bio-Rad AS 100" HRLC automatic sampling system with "Rheodine 7125 valve", "Bio-Rad 1350" HPLC pump and "Bio-Rad 1640" electrochemical detection. Chromatographic data were calculated using the "Chrome Line V 4.20" HPLC software. Amperometric detection was done at 0.6 V. The duration of chromatographic separation was 10 min.

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS®, version 17.0). Basic descriptive statistics included means, standard deviations, ranges and percentages. Differences between groups were compared with parametric *t*-test because data had a Gaussian distribution. Correlation analysis was processed *via* the Spearman method. Values at the $P \leqslant 0.05$ level were considered statistically significant.

RESULTS

The study included 11 (33.3%) female and 22 (66.7%) male patients, mean age of 52.32 (SD ± 11.55) years. The most common cause of liver cirrhosis was alcohol-in 15 (45.4%) cases. The incidence of posthepatitic cirrhosis was lower; HCV-8 (24.2%), HBV-5 (15.1%), while autoimmune diseases were quite rare-in 5 (15.1%) patients.

Splenomegaly was detected in 28 (84.8%) patients with liver cirrhosis. An average longitudinal splenic diameter was 17.5 ± 3.57 cm, and transversal diameter was 6.8 ± 1.77 cm, which was significantly different in relation to the controls, in whom an average longitudinal diameter was 10.21 ± 1.65 cm and transversal diameter was 3.03 ± 0.87 cm (*t*-test, $P < 0.05$).

There was a highly significant difference between the platelet count in the studied groups of patients ($t = -9.779$, $P < 0.01$).

The mean plasma free serotonin level was much higher in liver cirrhosis patients than in healthy controls (219.0 ± 24.2 nmol/L *vs* 65.4 ± 18.7 nmol/L; *t*-test, $P < 0.0001$).

There was no significant correlation between serotonin concentration in plasma and the platelet count according to Spearman coefficient of correlation ($r_s = 0.158$, $P > 0.05$).

Esophageal varices were not detected in 5 (15.1%) patients, grade Ⅰ-Ⅱ varices were detected in 9 (27.2%), grade Ⅱ-Ⅲ in 15 (45.4%) and the remaining 4 patients (12.1%) were grade Ⅳ. Gastric fundal varices were found in 7 (21.2%) patients, out of whom 2 had Ⅰ-Ⅱ, 4 had grade Ⅱ-Ⅲ esophageal varices, and one patient had Ⅳ degree varices.

Spearman's rank correlation verified a statistically significant correlation between the platelet count and varices size ($r_s = -0.479$, $P < 0.05$).

Spearman's rank correlation verified no significant difference between the serotonin concentration in plasma in relation to the size of esophageal varices (r_s = -0.217, P > 0.05). However, the mean plasma free serotonin level was higher in patients with esophageal varices than in patients without varices (t = -2.301, P < 0.05). Furthermore, the correlation of plasma serotonin concentration and fundal varices was highly significant (r_s = -0.601, P < 0.01). Also, we proved that the mean plasma free serotonin level was much higher in patients who had esophageal and gastric fundal varices than in patients who had only esophageal varices (t = -5.862, P < 0.01).

DISCUSSION

Different factors may affect the concentrations of circulating serotonin in liver cirrhosis, such as: impaired serotonin catabolism due to higher activity of the monoamino oxidases; impaired metabolism of tryptophane as a serotonin precursor; platelet sequestration in the spleen and/or platelet activation[11]. In addition, 5-HT as well as other vasoactive substances synthesized in the gastrointestinal tract *via* porto-systemic collaterals bypass the liver and directly enter the systemic circulation[12].

In the study of Beaudry *et al*[13], in 1994, the whole-blood serotonin levels were significantly lower in 30 patients with cirrhosis than in the age-matched controls, and no correlation was found between these levels and the severity of cirrhosis. However, in the same study the unconjugated plasma serotonin levels, an indication of the active form of serotonin, were significantly higher in patents with cirrhosis than in the controls.

In our previous study, free or unconjugated serotonin levels were investigated. The levels of free serotonin were higher in patients with liver cirrhosis than in healthy subjects[14].

In the study of Vorobioff *et al*[15], in 1989, it was confirmed that the application of ketanserin and ritanserin (serotonergic receptor inhibitors) caused the lowering of portal hypertension in patients with liver cirrhosis. The authors reported that the spleen congestion in liver cirrhosis brought about the platelet breakdown. Free serotonin, released in the sinusoidal spaces of the spleen, induced by S-2 receptor produced an intense vasoconstricting response in portal circulation which led to maintenance and elevation of the portal pressure. Moreover, it was documented that the reaction of the isolated mesenteric vein in rats with portal hypertension to 5-HT was hypersensitive, which was additional evidence of the role of this substance in pathogenesis of portal hypertension.

In our study, the correlation of unconjugated serotonin concentration (active form of serotonin) in plasma and varices of the gastric fundus was highly significant while the plasma unconjugated serotonin concentration did not correlate with the size of the esophageal varices. Moreover, mean longitudinal and transversal diameters of the spleen in patients was significantly higher as compared to controls.

The spleen has a crucial role in pathogenesis and maintenance of portal hypertension[16,17]. In portal hypertension, the anatomic changes of the spleen (pulp hyperplasia, congestion and fibrosis) and specific vascularization affect the hemodynamics of the splenic circulation[18].

Our finding may be accounted for different porto-systemic collateral pathways in esophageal and fundal varices as well as valuable flow changes in the left part of the portal venous system. Perisic *et al*[19] reported, in 2005, that the splenic vein flow in patients with liver cirrhosis was slower in comparison with healthy controls. In addition, in healthy controls, the splenic vein flow was significantly slower than in the portal vein. However, in patients with liver cirrhosis splenic vein flow was significantly faster than in the portal vein, probably because of the splenic venous congestion and compensatory hemodynamic mechanisms of the spleen.

Gastric varices are drained through the short gastric veins into the splenic vein. Serotonin released by platelet sequestration in the enlarged spleen reaches the lienal vein where the blood flow is faster than in the portal vein, and directly, *via* short gastric veins, it enters the fundal gastric veins, leading to vasoconstriction.

Our conclusion is that free serotonin is significant in pathogenesis of portal hypertension especially in development of gastric fundal varices which may have clinical value in use of serotonin receptor blockers in these patients.

COMMENTS

Background

In acute and chronic hepatic insufficiency, the serotonin system changes lead to development of hepatic encephalopathy, portal hypertension and hyperdynamic circulation. Portal hypertension has been increasingly regarded as a multi-organ disease with complex blood flow changes in systemic and splanchnic vascular network. The hepatic stellate cell (HSC) has a significant position in sinusoid for regulation of portal flow and during liver damage serotonin binds to receptors [5-hydroxytriptamin (5-HT)$_{2A}$, 5-HT$_{1B}$, 5-HT$_{1F}$ and 5-HT$_7$] which are expressed on HSC and hepatocytes, which additionally interferes with HSC proliferation.

Research frontiers

Free serotonin, released in the sinusoidal spaces of the spleen, induced by S-2 receptor, produces an intense vasoconstricting response in the portal circulation, which leads to maintenance and elevation of portal pressure. The highlight of our study was to determine to what extent a free serotonin concentration in plasma has an effect on development of esophageal and gastric fundal varices.

Innovations and breakthroughs

In the study of Vorobioff *et al*, in 1989, it was confirmed that the application of ketanserin and ritanserin (serotonergic receptor inhibitors) caused the lowering of portal hypertension in patients with liver cirrhosis. Moreover, it was documented that the reaction of the isolated mesenteric vein in rats with portal hypertension to 5-HT was hypersensitive, which was additional evidence of the role of this substance in pathogenesis of portal hypertension. In this study, the correlation of unconjugated serotonin concentration (active form of serotonin) in plasma and varices of the gastric fundus was highly significant while the plasma unconjugated serotonin concentration did not correlate with the size of the esophageal varices. The authors' finding may be accounted for by different porto-systemic collateral pathways in the esophageal and fundal varices as well as valuable flow changes in the left part of the portal venous system.

Applications

The conclusion is that free serotonin is significant in pathogenesis of portal hypertension especially in development of gastric fundal varices. This may have clinical value in use of serotonin receptor blockers in these patients.

Terminology

Portal hypertension: Portal hypertension (> 10 mmHg) most commonly results from increased resistance to portal blood flow. Cirrhosis is the most common

cause of portal hypertension. One of the major clinical manifestations of portal hypertension includes life-threatening hemorrhage from gastrointestinal varices. Serotonin: Serotonin is a vasoactive substance, synthesized by the intestinal enterochromaffin cells, which is actively incorporated into platelets and stored in platelet dense-storage granules.

Peer review

The manuscript reports that free serotonin is significant in pathogenesis of portal hypertension especially in development of gastric fundal varices which may have clinical value in use of serotonin receptor blockers in these patients.

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BRIEF ARTICLE

Value of duplex doppler ultrasonography in non-invasive assessment of children with chronic liver disease

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Abstract

AIM: To investigate the value of duplex Doppler ultrasonography (US) in the assessment of the hemodynamics of the portal and hepatic veins in a cohort of children with chronic liver disease (CLD) and to detect any relationship between the US changes, etiology and severity (or stage) of CLD.

METHODS: We prospectively enrolled 25 children with biopsy-proven CLD. Thirteen had cirrhosis (aged 8.9 ± 2.0 years) and 12 had chronic hepatitis (aged 9.3 ± 2.3 years). Gray scale and color-coded duplex Doppler US were performed for all, as well as 30 healthy age and sex-matched controls. Findings were correlated with clinical, laboratory and histopathological characteristics.

RESULTS: Prominent caudate lobe was detected in 100% of cirrhotics, but none of the chronic hepatitis or controls. Thickened lesser omentum and loss of the triphasic waveform of the hepatic vein were present in 69.2% and 53.8% of cirrhotics *vs* 33.3% and 8.3% of chronic hepatitis respectively. Portal vein flow velocity was significantly lower ($P < 0.0001$) and the congestion index was significantly higher ($P < 0.005$) in both patient groups compared to controls. Child-Pugh's staging showed a positive correlation with both abnormal hepatic vein waveform and direction of portal blood flow; and a negative correlation with both hepatic and portal vein flow velocities. No correlation with the etiology of CLD could be detected.

CONCLUSION: Duplex Doppler added to grayscale US can detect significant morphologic and portal hemodynamic changes that correlate with the severity (stage) of CLD, but not with etiology.

Key words: Chronic hepatitis; Chronic liver disease; Cirrhosis; Doppler; Grayscale; Pediatrics; Ultrasound

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INTRODUCTION

The search for a non-invasive biochemical or imaging marker for the severity (stage) and/or etiology of chronic liver disease (CLD) in adults, as well as children, is in extremely active nowadays. Real time ultrasonography (US) has become an integral part of the non-invasive evaluation of the liver in many clinical settings in adults. Color-coded duplex Doppler information regarding the presence or absence of flow and the direction and velocity of that flow can be obtained non-invasively, rapidly and relatively inexpensively[1]. In spite of being evaluated since 1983[2]; the accuracy, sensitivity and specificity of duplex Doppler imaging as a non-invasive diagnostic and prognostic modality for liver cirrhosis, and its correlation to the histopathologic findings as well as the degree of functional impairment of the liver, remains controversial and is still debated by many investigators[3]. Adding duplex Doppler evaluation in numerous studies has clarified the role of this modality in the evaluation of various CLD in adults and children, including liver cirrhosis[4-7]; portal hypertension[8-12]; presence or absence of esophageal varices[13], noninvasive diagnosis of the degree of hepatic fibrosis[14,15], assessment of the portal venous blood flow in cystic fibrosis[16], perioperative monitoring in orthotropic liver transplantation (LTx)[17], prediction of the severity of veno-occlusive disease and assessing its prognosis[18], assessment of the functional hepatic flow and total hepatic flow[19] and investigating the effect of fatty infiltration of the liver on the Doppler waveform pattern in the hepatic veins of obese children[3,20]. Other studies evaluated Doppler US measurement of the blood flow in the hepatic artery, hepatic veins and portal vein as a noninvasive indicator of disease severity in children who had undergone Kasai portoenterostomy for extrahepatic biliary atresia[21] and those who had CLD of unknown etiology[22]. However in the setting of LTx, serial intra- and post-operative Doppler US has largely been accepted as a useful technique for making an early diagnosis of abnormal hemodynamics of the graft circulation. Furthermore, intraoperative Doppler US is used to assess the reconstructed vessels objectively in order to reduce the incidence of vascular complications following LTx[6] and it is gradually replacing the more invasive angiographic techniques[23].

The aim of this study was to investigate the value of abdominal color-coded duplex Doppler US when added to the conventional grayscale scanning in the non-invasive assessment of the splanchnic morphology, as well as hemodynamics of the portal and hepatic veins in a cohort of Egyptian children suffering from CLD. We aimed also to detect any relationship between the US changes, etiology and severity (or stage) of the CLD.

MATERIALS AND METHODS

Materials and methods

We prospectively enrolled 25 children with CLD from the Pediatric Hepatology Unit at Cairo University Children Hospital, Cairo, Egypt. Thirteen patients (group 1) were diagnosed with established cirrhosis (7 girls and 6 boys) with a mean age of 8.9 ± 2.0 years, and 12 (group 2) were diagnosed with chronic hepatitis without cirrhosis (6 girls and 6 boys) with a mean age of 9.3 ± 2.3 years. Thirty healthy child relatives of the patients (13 girls and 17 boys) with a mean age of 8.1 ± 2.2 years were included as a control group. All patients were subjected to: (1) Careful interrogation and thorough physical examination for signs of CLD (jaundice, palmar erythema, bleeding diathesis, hand tremors, hepatomegaly, splenomegaly, ascites and edema); (2) biochemical tests of liver functions; (3) serological markers of viral and autoimmune hepatitis; (4) testing for inborn errors of metabolism when indicated; and (5) percutaneous liver biopsy using the Menghini aspiration technique for histopathological diagnosis, grading and staging of the CLD.

Patients, as well as controls, underwent conventional grayscale US using Toshiba® Sonolayer 2000 apparatus (Toshiba Corporation, Tokyo, Japan) equipped with 3.5 and 5 MHz convex linear transducers. Examination included liver size and echo pattern, portal vein diameter, splenic size and echo pattern, thickness of the lesser omentum in comparison to the aorta as well as detection of ascites. Both patients and controls also underwent color-coded duplex Doppler examination using Toshiba® Sonolayer SSH-60A apparatus (Toshiba Corporation, Tokyo, Japan) with a low frequency (3.5 MHz) transducer, in order to optimize the return of Doppler signals from deeper-lying tissues.

After being fasted overnight for a minimum of 8 h, all children were examined in the supine position. A low pulse repetition frequency was used initially, with manual adjustment when aliasing occurred. Approach for the vein of interest was selected to keep the beam vessel (angle 0) always less than 60°. Doppler recording of the hepatic veins was initially examined using a transverse sub-xiphoid approach, with the probe slightly cephalad. The right intercostal approach was used to obtain a longitudinal view of the middle hepatic vein. Every child was asked to stop breathing for a few seconds, in deep inspiration, during examination to avoid motion artifacts. Measurements were obtained from the hepatic veins at least 2 cm from the confluence with the inferior vena cava, to reduce the possible influence of the changes of flow pattern in the inferior vena cava on hepatic veins hemodynamics. We classified the hepatic vein Doppler waveforms according to Gorka *et al*[24] into: normal triphasic, abnormal biphasic or monophasic, and those with loss of the reverse-flow. Mean flow velocity of the middle hepatic vein was also measured in cm/s in all patients and controls.

The portal vein was examined from an anterior abdominal subcostal and/or right intercostal approach and scanned longitudinally throughout its entire length. Measurements were obtained in the middle segment between the splenoportal junction and the intrahepatic bifurcation (1-2 cm before the bifurcation). It was examined at a standard point for the diameter, patency, presence or absence

Table 1 Patients demographic and clinical data *n* (%)

Diagnosis	Group 1 (cirrhosis)	Group 2 (chronic hepatitis)
Symptoms		
Hematemesis	2 (15.4)	3 (25)
Jaundice	4 (38.5)	3 (25)
Dark urine	5 (38.5)	4 (33.3)
Abdominal distension	8 (61.7)	8 (66.7)
General physical signs		
Pallor	5 (38.5)	3 (25)
Jaundice	5 (38.5)	4 (33.3)
Lower limb edema	6 (46.2)	4 (33.3)
Abdominal signs		
Hepatomegaly	7 (53.8)	9 (75)
Splenomegaly	10 (76.9)	4 (33.3)
Ascites	5 (38.5)	3 (25)

of intraluminal echogenic material, direction of flow within the vein and blood flow velocity in cm/s.

The congestion index (CI) is determined by duplex Doppler US. It is the ratio between the cross-sectional area of the portal vein in cm^2 and the blood flow velocity in that vein in cm/s, and calculated using the following formula: CI = cross sectional area/blood flow velocity[25,26].

The cross sectional area was calculated using the following formula: Cross sectional area = $\pi \times (d^2/4)$; d = diameter of portal vein in cm; and $\pi = 3.14$[27].

Statistical analysis

All data were statistically analyzed using independent samples test, χ^2 test, post-Hock test and Armitage[28]. Probability (*P* value) was considered significant if $P < 0.05$.

RESULTS

Group 1 included 13 children with established cirrhosis (5 of metabolic etiology and 8 viral hepatitis C or B) and group 2 included 12 with chronic hepatitis and no cirrhosis (5 viral hepatitis C or B and 7 autoimmune). Table 1 shows the demographic and clinical data of the 2 groups. Biochemical tests of liver function showed no significant difference between either group (similar letters meant no significant difference, while dissimilar letters meant a significant difference, the *P* value is significant).

A prominent caudate lobe (as an important US sign) was found in 100% of patients with cirrhosis and none of patients with chronic hepatitis or controls. Thickened lesser omentum (i.e. lesser omentum: aortic diameter ratio > 1:1.7 in children[29]) was present in 69.21% of cirrhotic patients in comparison to 33.3% of patients with chronic hepatitis. Loss of the normal triphasic oscillation of the hepatic vein waveform was detected in 53.8% of group 1 in comparison to 8.3% of group 2 and none of the controls. Abnormal direction of portal blood flow was detected in 46.2% of group 1 and 25% of group 2, and none of the controls (Figures 1 and 2).

Hepatic vein flow velocity showed non-significant negative correlation with liver size (r = -0.125) and weak

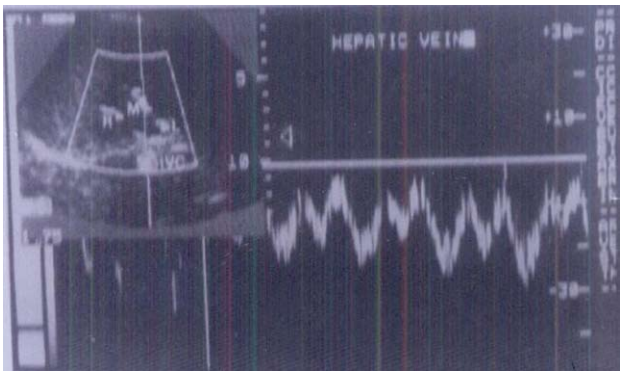

Figure 1 Triphasic waveform pattern of normal hepatic vein.

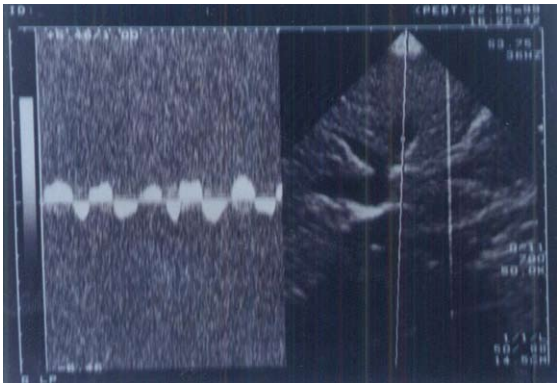

Figure 2 Abnormal waveform pattern of hepatic veins in a cirrhotic liver.

but significant correlation with both splenic size (r = -0.374) and portal vein diameter (r = -0.304) (Table 2).

Portal vein flow velocity had weak significant negative correlation with liver size (r = -0.431) and powerful significant negative correlation with both splenic size (r = -0.699) and portal vein diameter (r = -0.743, Figure 3).

CI had a weak significant negative correlation with liver size (r = -0.431) and powerful significant negative correlation with both splenic size (r = -0.699) and portal vein diameter (r = -0.743).

According to Child-Pugh's[30] classification, 4 (31%) of our cirrhotic patients were Class A; 4 (31%) Class B and 5 (38%) Class C. Those classes showed a powerful significant positive correlation with both abnormal hepatic waveform and abnormal direction of portal blood flow. Also there was a powerful significant negative correlation with both hepatic vein and portal vein flow velocity (r = -0.785 and -0.688, respectively, Figure 4) and weak significant positive correlation with CI (r = -0.595).

Analysis of the US findings according to the etiological categories (metabolic, viral or autoimmune CLD) did not reveal any significant correlations.

DISCUSSION

In the present study we tried to correlate the splanchnic morphological and hemodynamic parameters of the portal and hepatic veins with the severity of hepatic affection, as

Table 2 Grayscale and Doppler ultrasonography measurements in the studied groups (mean ± SD)

	Controls	Group 1	Group 2	*P* value
Liver size in cm	8.55 ± 0.69	10.19 ± 2.33[b]	12.2 ± 2.02[b,d]	< 0.01
Splenic size in cm	8.43 ± 0.92	11.82 ± 1.30[b]	10.10 ± 1.50[b,d]	< 0.01
Portal vein diameter (cm)	0.61 ± 0.13	1.16 ± 0.23[b]	0.95 ± 0.22[b,d]	< 0.01
Hepatic vein flow velocity (cm/s)	23.1 ± 2.09	21.84 ± 1.90	22.41 ± 2.02	> 0.05
Portal vein flow velocity (cm/s)	31.1 ± 3.92	21.23 ± 3.30[b]	22.22 ± 1.23[b]	< 0.01
Congestion index	4.61 ± 5.75[d]	9.50 ± 1.60	4.97 ± 1.14[d]	< 0.01

[b]$P < 0.01$ *vs* controls; [d]$P < 0.01$ *vs* group 1.

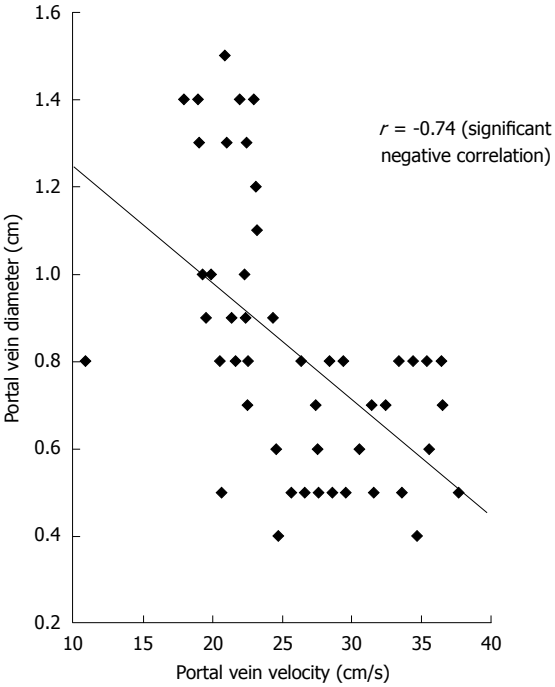

Figure 3 Correlation between portal vein flow velocity and portal vein diameter.

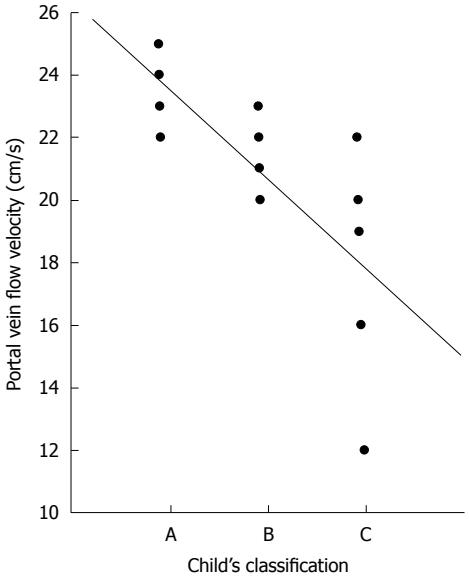

Figure 4 Correlation between Child-Pugh's classification and portal vein velocity.

evaluated by liver histopathology and Child-Pugh's classification of cirrhosis[30]. Prominent caudate lobe was a constant finding in all our patients with cirrhosis and not found in chronic hepatitis. The prominence of the caudate lobe in cirrhosis results from marked hyperplasic changes in the regenerative nodules with no cellular or structural atypia. The density of the regenerative hepatocytes becomes much higher, the quantity of bound water larger and free water smaller, increasing the US signals reflected. The reason why only the caudate lobe shows such huge hyperplasia in cirrhosis remains unclear[31].

Thickened lesser omentum (i.e. lesser omentum: aortic diameter more than 1:1.7[29]) is highly-suggestive of portal hypertension and the presence of esophageal varices in children, and allows the detection of portal hypertension earlier than detection of collaterals by Doppler and even earlier than clinical signs[32]. In our study there was a significant increase of lesser omentum thickness in cirrhotic patients than chronic hepatitis.

Koda *et al*[8], in 1996 described the decrease in portal vein flow velocity with the progress of chronic hepatitis as a sensitive indicator and a useful test with close correlation with the histological degree of liver fibrosis. Schneider *et al*[33], combined portal vein velocity with hepatic artery pulsatility index as a reliable, non-invasive evaluation of patients with liver cirrhosis. On the other hand Dinç *et al*[34], reported that portal vein flow velocity and portal vein flow volume alone are not useful parameters for discriminating cirrhotic patients from healthy subjects. In our study we did not measure the hepatic artery pulsatility index; however values of portal vein flow velocity were significantly lower in patients compared to controls and significantly lower in the group with liver cirrhosis than the chronic hepatitis. A significant correlation was also detected between both splenic size and Child-Pugh's class and the portal flow velocity; the lower the velocity, the larger the splenic size and the worse the Child-Pugh's class. Therefore in children with cirrhosis, portal flow velocity might be correlated with the severity of portal hypertension and the severity of liver parenchymal dysfunction as worsening Child-Pugh's class was associated with lower portal vein flow velocity conforming to reports in adults[35].

In the present study CI was significantly higher in the group with liver cirrhosis than both the chronic hepatitis group and the controls. CI showed no significant differ-

ence between chronic hepatitis group and controls. CI showed a positive correlation with Child-Pugh's class of cirrhotics. CI was reported to be a significant parameter in the evaluation of the risk of bleeding varices and prognosis of patients with liver cirrhosis, while in chronic hepatitis patients it was found to be similar to healthy controls and was not related to the grade of hepatic inflammation[36].

Loss of the normal triphasic oscillation of the hepatic vein waveform was detected in 53.8% of our cirrhotics, in comparison to 8.3% of chronic hepatitis and none of the controls. Bolondi *et al*[37], Ohta *et al*[38] and Arda *et al*[39], reported the loss of triphasic oscillation even in early-stage chronic parenchymal liver disease (Child-Pugh's class A).

In conclusion, grayscale and color-coded duplex Doppler US are very valuable, non-invasive diagnostic modalities in children with CLD. They could detect splanchnic morphological and portal hemodynamic changes that could be correlated to the degree of liver parenchymal affection but not to the etiology of the CLD. Therefore we recommend their wider application in the assessment of children with CLD.

COMMENTS

Background

The search for a non-invasive biochemical or imaging marker for the severity (stage) and/or etiology of chronic liver disease (CLD) in adults as well as children is in extremely active nowadays. Real time ultrasonography (US) has become an integral part of the non-invasive evaluation of the liver in many clinical settings in adults. Color-coded duplex Doppler information regarding the presence or absence of flow and the direction and velocity of that flow can be obtained non-invasively, rapidly and relatively inexpensively.

Research frontiers

The aim of the research is to investigate the value of duplex Doppler US in the assessment of the hemodynamics of the portal and hepatic veins in a cohort of children with CLD, and to detect any relationship between the US changes and etiology and severity (or stage) of CLD.

Innovations and breakthroughs

In the present study, the authors tried to correlate the splanchnic morphological and hemodynamic parameters of the portal and hepatic veins with the severity of hepatic affliction. In this study, the authors found that the values of portal vein flow velocity were significantly lower in patients compared to controls and significantly lower in the group with liver cirrhosis than the chronic hepatitis group. A significant correlation was also detected between both splenic size and Child-Pugh's class and the portal flow velocity; the lower the velocity, the larger the splenic size and the worse the Child-Pugh's class. Therefore in children with cirrhosis, portal flow velocity might be correlated with the severity of portal hypertension and the severity of liver parenchymal dysfunction.

Applications

In conclusion, grayscale and color-coded duplex Doppler US are very valuable, non-invasive diagnostic modalities in children with CLD. They could detect splanchnic morphological and portal hemodynamic changes that could be correlated to the degree of liver parenchymal affection but not to the etiology of the CLD. Therefore the authors recommend their wider application in the assessment of children with CLD.

Peer review

This article well documented that the grayscale and color-coded duplex Doppler US are very valuable for non-invasive diagnostic modalities in children with CLD, and this will interest the readers.

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BRIEF ARTICLE

Pegylated interferon α-2b up-regulates specific CD8+ T cells in patients with chronic hepatitis B

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Abstract

AIM: To investigate the effect of pegylated interferon (IFN) α-2b on specific CD8+ T lymphocytes in patients with chronic hepatitis B (CHB).

METHODS: Twenty-one patients with CHB were treated with pegylated IFN α-2b. Periphery blood mononuclear cells were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation (density: 1.077 g/L, Pharmingen) at weeks 0, 4, 8, 12, and 24, respectively. Frequency of circulating hepatitis B virus (HBV) epitope-specific CD8 T cells was detected by flow cytometry. Cytokines were detected by cytometric bead assay.

RESULTS: The frequency of circulating HBV core or env-specific CD8 T cells was higher ($P < 0.05$), the number of HBV core specific CD8 T cells was greater at week 24 ($P < 0.05$), the level of Th1-type cytokines [interleukin (IL)-12, tumor necrosis factor-α, and IFN-γ] was higher, while that of Th2-type cytokines (IL-4, IL-6, and IL-10) was lower in responders than in non-responders ($P < 0.05$) after pegylated IFN α-2b treatment. The IL-6 level was correlated with HBV DNA ($r = 0.597$, $P = 0.04$), while the inducible protein-10 (IP-10) level was correlated with serum alanine aminotransferase (ALT) ($r = 0.545$, $P = 0.005$). The IP-10 level at week 8 after pegylated IFN α-2b treatment could predict the normalization of ALT in CHB patients (positive predict value = 56%, negative predict value = 92%).

CONCLUSION: Pegylated IFN α-2b can enhance the immune response of CHB patients by increasing the frequency of HBV specific CD8+ T cells and regulating the Th1/Th2 cytokines.

Key words: Chronic hepatitis B; Pegylated interferon α-2b therapy; Immune response; Cytokine

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INTRODUCTION

More than two billion people have been infected with hepatitis B virus (HBV) and chronic HBV infection affects about 400 million people worldwide[1,2]. Chronic hepatitis B (CHB) is a chronic inflammatory liver disease,

which can progress to end-stage liver diseases, such as cirrhosis and hepatocellular carcinoma.

Adaptive immunity plays a central role in the pathogenesis of chronic HBV infection, and it is crucial to understanding the behavior of T cell response for the design of effective strategies for the control of HBV infection[3-5]. Different studies in chronic and early acute phases of HBV infection suggested that the functional impairment of HBV-specific cell-mediated immune response plays an important role in HBV persistence[6-14]. Moreover, recent studies showed that both positive and negative signals regulate the antigen-specific T cell function and are important for the better outcome of patients with HBV infections[15-17].

Pegylated interferon (IFN) α-2b can modulate and reduce antiviral function of CHB patients by enhancing their immune responses. However, the exact effect of pegylated IFN α-2b on the immune responses of patients with HBV infections remains unclear. The present study was designed to investigate the effect of pegylated IFN α-2b on HBV specific CD8+ T cells and secretion of cytokines in CHB patients.

MATERIALS AND METHODS

Patients and study design

Twenty-one consecutive CHB patients (17 males and 4 females) at the age of 20-39 years (mean 25 years), admitted to our hospital from January 2008 to May 2009 were included in this study. Diagnosis of HBV infection was established as previously described[18]. Clinical data and characteristics of the patients are summarized in Table 1. The patients were treated with pegylated IFN α-2b (PegIntron from Schering-Plough), at the dose of 0.5-1 μg/kg of body weight, once a week for 24 wk. Clinical and laboratory data about the patients were detected before treatment, or at weeks 4, 8, 12, and 24 after treatment. Patients co-infected with HBV and HCV or with detectable antibodies against hepatitis delta virus or against human immunodeficiency virus were excluded, as were those with other causes of liver disease, including alcohol abuse. No patient had decompensated liver disease (evidence or history of ascites, variceal bleeding, hepatic encephalopathy or jaundice).

Isolation of peripheral blood monouclear cells

Peripheral blood monouclear cells (PBMC) were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation (density: 1.077 g/L, Pharmingen). Blood was two-fold diluted with RPMI 1640 medium containing 300 μg/mL L-glutamin, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum, then added into the isovolumic Ficoll, centrifuged for 400 × *g* at 21℃ for 35 min. The cells were washed twice with phosphate buffered saline (PBS).

Human leukocyte antigen-A2 typing

One hundred microliters of fresh heparinized blood (100) was incubated with human leukocyte antigen-A2 primary antibody for 30 min. Erythrocytes were lysed with an erythrocyte lysate at 37℃, washed with PBS, and then incubated with secondary antibody, washed again and analyzed on Becton Dickinson FACS (Becton Dickinson, USA).

Analysis of HBV epitope-specific CD8+ T cells

Frequency of HBV epitope-specific CD8 T cells was detected by flow cytometry after incubated with HBV core18-27 tetramers (ProImmune, Oxford, UK) and HBV env 335-343 pentamers (ProImmune, Oxford, UK). Freshly isolated PBMC were incubated with PE-labeled tetramer or pentamer in PBS (10% FCS) for 15 min at 37℃, washed once with PBS (1% FCS) and then incubated on ice for 30 min with FITC-anti-CD8 (ProImmune, Oxford, UK), washed twice with PBS, adjusted to 1×10^6 cells/vial, and fixed in 2% paraformaldehyde for analysis. About 1×10^6 PBMC were harvested and analyzed within the CD8 gate on Becton Dickinson FACS using the CELLQuest™ software.

Secretion of cytokines

Serum levels of interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ and inducible protein-10 (IP-10) in CHB patients were measured by cytometric bead assay (BD, USA) according to its manufacturer's instructions.

Serological assessment

Fasting serum levels of liver enzymes [alanine aminotransferase (ALT), aspartate aminotransferase] were measured with a Hitachi-7180 automatic biochemistry analyzer (Hitachi Inc., Japan) following the standard laboratory methods. HBV DNA was detected by real time polymerase chain reaction (Amplicor, Roche).

Statistical analysis

All data were analyzed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Descriptive baseline data were expressed as mean ± SD for continuous variables. Differences between groups were assessed using Kruskal-Wallis H for continuous variables. Spearman *P* test was performed for correlation analysis. The accuracy of serum factors for predicting virologic response was assessed using the receiver operating characteristic curve. $P < 0.05$ was considered statistically significant.

RESULTS

Frequency of circulating HBV epitope-specific CD8 T cells in CHB patients after pegylated IFN α-2b treatment

Circulating HBV epitope-specific CD8 T cells were detected 13 out of the 21 CHB patients (Table 1). The frequency of HBV core 18-27 tetramers+/CD8+ T cells at week 0 was 0.013 ± 0.002, which increased to 0.026 ± 0.015, 0.029 ± 0.019, 0.036 ± 0.025, and 0.045 ± 0.027, respectively, at weeks 4, 8, 12, and 24 after IFN α-2b treatment (Figure 1), with a significant difference between weeks 8 and 0, and between weeks 24 and 0 ($P < 0.05$). The frequency of HBV env 335-343 pentamers+/CD8+ T cells began to increase at week 8 with a significant difference between weeks 24 and 0 ($P < 0.05$). No significant difference was observed in frequency of HBV core and HBV env specific CD8 T cells.

Table 1 Clinical characteristics of chronic hepatitis B patients included in this study

Patient	Age (yr)/sex	HBV DNA (IU/L)	ALT (U/L)	Total bilirubin (mg/dL)	Albumin (g/dL)	Platelets ($\times 10^9$/L)	HBeAg	HBeAb	HBsAg	HBsAb	Genetype
1	27/M	201 000 000	143	9.1	43.7	113	+	-	+	-	C
2	25/M	160 000 000	147	12.9	47.4	167	+	+	+	-	C
3	30/M	471 000 000	205	11.9	44.3	127	+	-	+	-	C
4	21/M	32 200 000	123	10.8	45.8	181	+	-	+	-	C
5	21/M	186 000 000	148	14.2	44.1	110	+	-	+	-	C
6	38/F	30 800 000	347	18.1	45.5	126	+	-	+	-	C
7	20/F	29 000 000	171	10.0	44.9	284	+	-	+	-	C
8	20/M	143 000 000	-	13.6	48.0	248	+	-	+	-	B
9	20/F	621 000 000	112	13.4	48.5	170	+	-	+	-	C
10	23/M	597 000 000	196	18.9	43.2	201	-	+	+	-	C
11	20/F	2 910 000	98	15.6	48.0	137	+	+	+	-	C
12	38/M	63 700 000	206	11.1	51.2	142	+	+	+	-	C
13	28/M	134 000 000	138	9.0	45.9	174	+	+	+	-	C
14	25/M	237 000 000	93	16.9	51.3	130	+	-	+	-	B
15	39/M	1 190 000 000	122	18.2	50.6	166	+	-	+	-	C
16	36/M	8 820 000	170	22.9	47.1	169	+	-	+	-	C
17	25/M	655 000 000	90	20.9	46.8	161	+	-	+	-	B
18	25/M	157 000 000	164	24.3	47.3	209	-	+	+	-	C
19	20/M	290 000 000	124	11.9	47.8	140	+	-	+	-	B
20	23/M	655 000 000		19.9	44.6	194	+	-	+	-	B
21	28/M	15 300 000	237	14.5	45.3	154	+	-	+	-	C

HBV: Hepatitis B virus; ALT: Alanine aminotransferase; HBeAg: Hepatitis B e antigen; HBeAb: Hepatitis B e antibody; HBsAg: Hepatitis B surface antigen; HBsAb: Hepatitis B surface antibody.

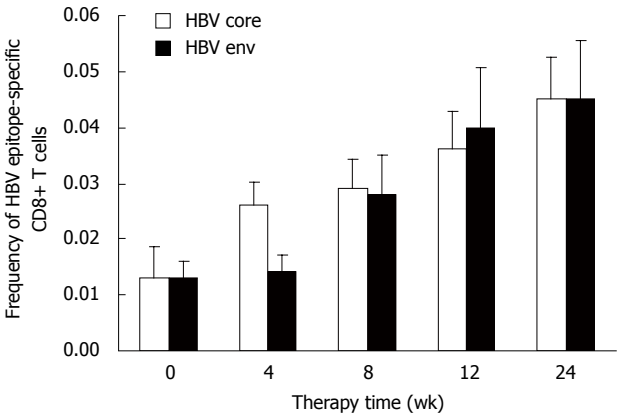

Figure 1 Frequency of hepatitis B virus epitope tetramer+/CD8+ T cell after pegylated interferon α-2b treatment. The frequency of hepatitis B virus (HBV) specific CD8+ T cells was increased connectively at weeks 4, 8, 12 and 24 after pegylated interferon α-2b treatment with no difference in frequency of HBV core specific CD8+ T cells and HBV env specific T cells.

To further analyze the effect of pegylated IFN α-2b on HBV-specific CD8 T cells, 13 patients were divided into responders (n = 7) and non-responders (n = 6). Responders were defined as their ALT returned to its normal level and their HBV DNA was decreased to over 2log, and/or their serum HBeAg was conversed. The frequency of HBV core18-27 tetramers+/CD8+ T cells was 0.014 ± 0.011, 0.029 ± 0.022, 0.029 ± 0.021, 0.067 ± 0.029, and 0.05 ± 0.025, respectively, in responders at weeks 0, 4, 8, 12 and 24 after treatment, which was higher than that in non-responders (0.012 ± 0.007, 0.018 ± 0.009, 0.028 ± 0.019, 0.025 ± 0.021 and 0.030 ± 0.01, respectively). No significant difference was found in frequency of HBV core specific CD8 T cells between responders and non-responders at baseline, even at weeks 4, 8, and 12 after treatment (Figure 2), with a significant difference observed at week 24 ($P < 0.05$, Figure 3). The frequency of HBV env specific CD8 T cells was higher in responders than in non-responders ($P < 0.05$, Figure 2).

Secretion of cytokines after pegylated IFN treatment and its correlation with virologic responses

The serum levels of IL-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-α, IFN-γ, IL-12, and IP-10 were measured at baseline, during the treatment and follow-up. The serum IL-2 level was very low in CHB patients, which was almost undetectable. The levels of Th1-type cytokines including IL-12, TNF-α and IFN-γ were increased while those of Th2-type cytokines including IL-4, IL-6 and IL-10 were decreased at week 48 after treatment (Figure 4). The baseline IP-10 level was increased from week 4 and decreased from week 48 after treatment.

The baseline IL-6 level was correlated with HBV DNA in responders (r = 0.597, P <0.05) but not with HBV DNA in non-responders. IL-10 was correlated with IL-6 (r = 0.762, P = 0.002), and IL-12 was correlated IFN-γ (r = 0.485, P = 0.026).

The IP-10 level was closely correlated with the serum ALT level not only in responders but also in non-responders (r = 0.545, P = 0.005, Figure 5), indicating that IP-10 level fluctuates with serum ALT level. The baseline IP-10 level was lower in patients with their ALT < 40 U/L than in those with their ALT > 40 U/L.

Predictability of IP-10

To determine whether IP-10 can predict the normalization of ALT (< 40 U/L) after peg-IFN α-2b treatment, receiver operating characteristic curve was plotted for IP-10. The

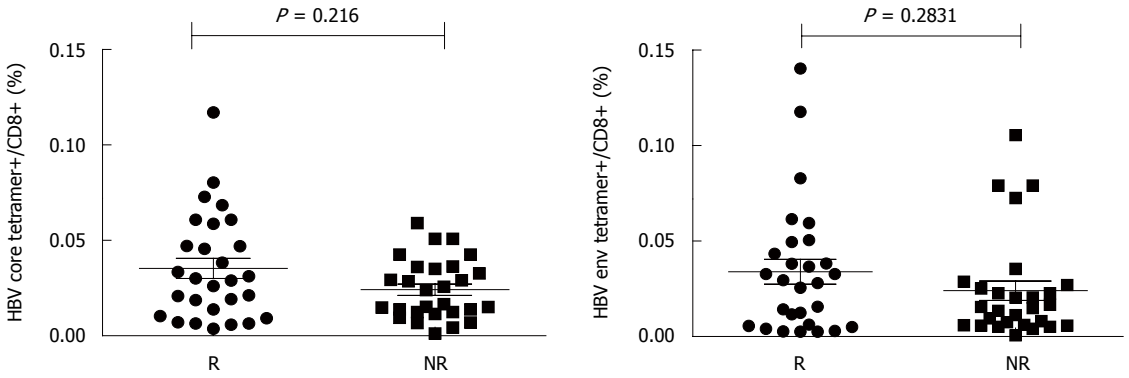

Figure 2 No correlation between increased hepatitis B virus epitope-specific CD8+ T cells and treatment outcome. The frequency of hepatitis B virus (HBV) core or env epitope-specific CD8+ T cells was higher in non-responders (NR) than in responders (R).

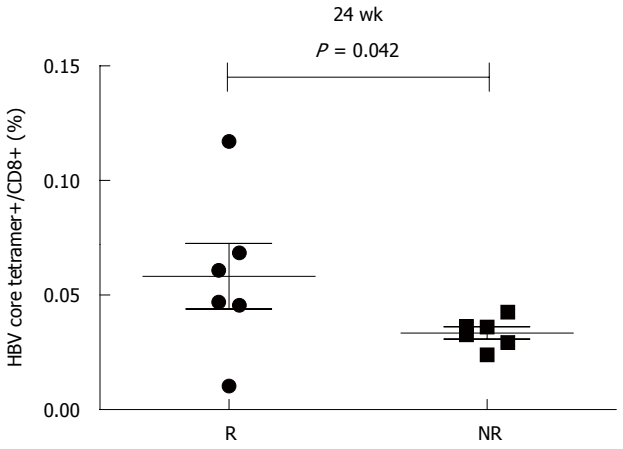

Figure 3 Correlation between increased hepatitis B virus specific T cells and treatment response 24 wk after therapy. The frequency of hepatitis B virus (HBV) core epitope-specific CD8+ T cells at week 24 was higher in responders (R) than in non-responders (NR).

IP-10 level at week 8 after treatment was predictable. The area under the curve was 0.741 ($P = 0.065$). A cutoff value of 437.78 was chosen. Correspondingly, the positive and negative predictive value was 56% and 92%, respectively (Table 2).

DISCUSSION

HBV has a high propensity to persist and several strategies have been developed for control of its evading from T cell responses, including the direct inhibitory effect of viral proteins on T cell responses and the emergence of escape mutations[19-21]. Moreover, HBV infection is more common in immune deficient individuals, such as infants, patients with cancer and those treated with steroid hormone, thereby can interfere with viral clearance by the innate immune system[22,23]. Inefficient innate responses and rapid spread of HBV may in turn delay and impair adaptive responses because of inefficient promotion of T cell priming by innate immunity and through T cell exhaustion induced by a rapidly increased viral load. However, the actual impact of exhaustion by persistent exposure to high antigen concentrations on virus persistence has only been partially defined.

Furthermore, two kinds of drugs (nucleoside analogs and IFN) are usually used in antiviral treatment of CHB

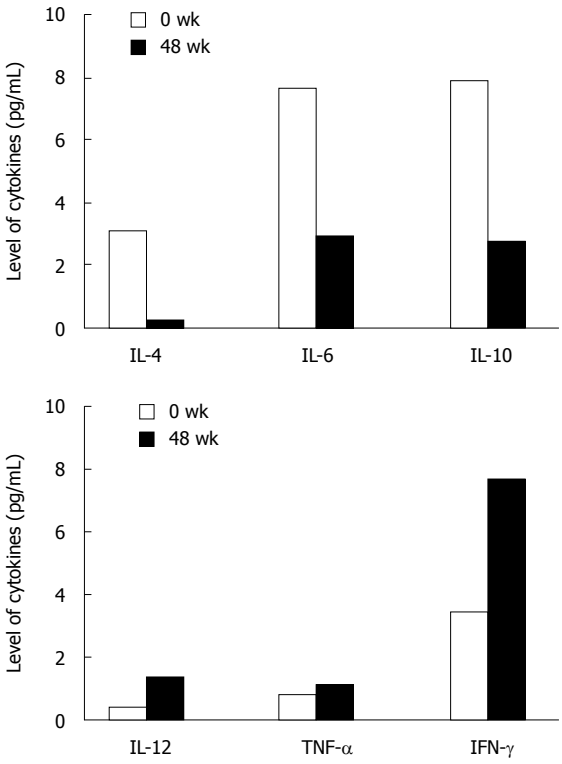

Figure 4 Level of cytokines after treatment. The levels of Th1-type cytokines [interleukin (IL)-12, tumor necrosis factor (TNF)-α and interferon (IFN)-γ] were higher, while the levels of Th2-type cytokines (IL-4, IL-6 and IL-10) were lower at week 48 after treatment.

patients. IFN is involved in numerous immune interactions during viral infection, as an inducer, regulator, and effector of both innate and adaptive antiviral systems. IFN-α and beta are produced rapidly due to viral factors, such as envelope glycoprotein, CpG DNA or dsRNA, and interact with cellular pattern-recognition receptors, such as mannose receptors, toll-like receptors, and cytosolic receptors[24]. In addition, IFN modulates both innate and adaptive immunity, ultimately resulting in an enhanced antiviral effector function.

In the present study, the frequency of HBV epitope-specific CD8+ T cells in peripheral blood was persistently increased at weeks 4, 8, 12 and 24 after peg-IFN α-2a treatment, while the number of HBV epitope-specific CD8 T cells in HBV core 18-27 tetramers and HBV env 335-343

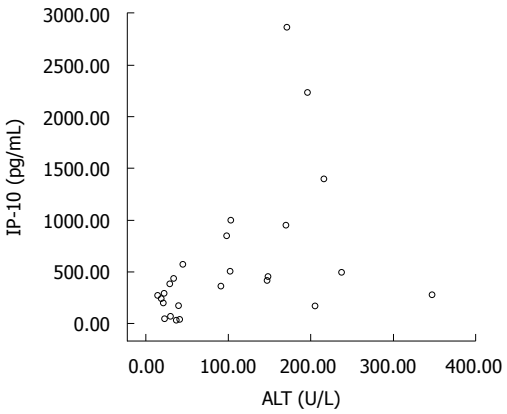

Figure 5 Positive correlation between alanine aminotransferase and inducible protein-10 levels ($r = 0.545$, $P = 0.005$). ALT: Alanine aminotransferase; IP-10: Inducible protein-10.

Table 2 Predictive value of serum inducible protein-10 levels 8 wk after treatment

IP-10 (pg/mL)	ALT < 40 U/L	ALT ≥ 40 U/L	Predictive value
< 437.78	5	4	PPV = 56%
≥ 437.78	1	11	NPV = 92%

IP-10: Inducible protein-10; ALT: Alanine aminotransferase; PPV: Positive predict value; NPV: Negative predict value.

pentamers was greater in responders than in non-responders after pegylated IFN α-2b treatment, suggesting that the therapeutic effect of pegylated IFN α-2b on HBV infection may be attributed to the elevated HBV-specific CD8 T cells, and that the immune response mediated by HBV-specific cells plays an important role in control of HBV. However, the frequency of HBV core 18-27 tetramers+/CD8+ T cells was higher than that of HBV env 335-343 pentamers+/CD8+ T cells after pegylated IFN treatment, suggesting that the HBV core epitope plays a more critical role in induction of a stronger immune response to HBV infection than to HBV env epitope. Pegylated IFN α-2b could enhance specific immune response of CHB patients. Further study should be performed with a large sample size.

Cytokines play an important role in immune modulation. Clearance of HBV infection is mediated by a strong polyclonal cellular response of both CTL and Th1 cells. Chronic HBV infection is caused mainly by an increased response of Th2 cells and impaired production of type 1 cytokines. IL-10, a Th2-type cytokine secreted by T-cells, activated B cells and monocytes, is a powerful inhibitor of Th1 activation and suppresses cell-mediated immunity in mice and humans[25,26]. Of the detected cytokines, Th2-type cytokines such as IL-4, IL-6 and IL-10, were altered conspicuously. After treatment, the level of Th2-type cytokines (IL-4 and IL-10) was down-regulated, thus confirming the immune recover potential of pegylated IFN α-2b, the level of IL-12 which can promote the differentiation of Th1-type cytokines was low, and the production of Th1-type cytokines was increased, indicating that the immune function of pegylated IFN α-2b can be achieved by regulating the balance of Th1/Th2 cytokines.

IL-6 is a multifunctional cytokine with both differentiation and growth-promoting effects for a variety of target cells. IL-6 is generally considered an important cytokine in the network of cytokines that regulate immune reactions and acute phase responses[27]. It was reported that IL-6 is correlated with liver fibrosis/cirrhosis[28] and is a cell attachment site for HBV[29]. In the present study, the IL-6 level was correlated with HBV DNA plasma only in responders.

IP-10, a chemotactic CXC chemokine of 77 aa in its mature form[30,31], can be produced by a variety of cells, including hepatocytes[32,33]. The correlation between IP-10 levels and necroinflammatory activity, as well as the high and low IP-10 levels before and after pegylated IFN α-2b treatment, may imply that IP-10 plays a role in the natural pathogenesis of HBV-induced liver damage[34]. It was reported that the baseline IP-10 level can predictive the response of CHB patients to HCV treatment, and is correlated with liver inflammation and fibrosis[35,36]. In this study, the baseline IP-10 level in CHB patients could predict the normalization of ALT after pegylated IFN α-2b treatment.

In conclusion, given the importance of protective T cell responses in control of HBV, the correlation between immunomodulatory molecules and pegylated IFN α-2b treatment in restoration of the immune responses of antiviral T cells are highly desirable. Pegylated IFN α-2b therapy can enhance the immune response of CHB patients by influencing the production of cytokines. IP-10 can potentially predict the normalization of ALT, which is correlated with liver damage. Further study is needed with a large sample size.

ACKNOWLEDGMENTS

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COMMENTS

Background

More than two billion people have been infected with hepatitis B virus (HBV) and chronic HBV infection affects about 400 million people worldwide. Two kinds of drugs [nucleoside analogs and interferon (IFN)] are mainly used in treatment of chronic hepatitis B (CHB) patients. IFN is involved in numerous immune interactions as an inducer, regulator, and effector in treatment of viral infections. Cytokines play an important role in immune modulation. Clearance of HBV infection is mediated by a strong polyclonal cellular response of both CTL and Th1 cells. Chronic HBV infection is caused mainly by an increased response of Th2 cells and impaired production of type 1 cytokines. Inducible protein 10 (IP-10) is a chemotactic CXC chemokine of 77 aa in its mature form.

Research frontiers

IFN-α and β are produced rapidly due to viral factors, such as envelope glycoproteins, CpG DNA or dsRNA, and interact with cellular pattern-recognition receptors, such as mannose receptors, toll-like receptors, and cytosolic receptors. IP-10 can be produced by a variety of cells, including hepatocytes. The results of this study show that the baseline IP-10 level can predict the response of patients with HBV infection to its treatment with pegylated IFN α-2b.

Innovations and breakthroughs

The present study demonstrated the correlation between pegylated IFN α-2b treatment and HBV-specific T lymphocytes. In addition, the effect of pegylated IFN α-2b on HBV infection could be achieved by balancing the production of Th1/Th2 cytokines and IP-10 could predict the outcome of patients with HBV infection after pegylated IFN α-2b treatment.

Applications

In this study, pegylated IFN α-2b could up-regulate HBV epitope specific CD8+ T cells. The specific cellular immune response could control HBV. IP-10 serum

level could predict the outcome of patients with HBV infection after pegylated IFN α-2b treatment, thus providing a new index for the treatment of HBV infection. Pegylated IFN α-2b may be used as a novel strategy for the treatment of HBV infection by regulating the cytokines.

Terminology

Human leukocyte antigen (HLA) typing is a method to define the HLA+ and HLA+ blood for studied subjects. Flow cytometry is used to define the HBV epitope specific CD8+ T lymphocytes. Cytometric bead assay is a new technique for detecting serum concentration of cytokines.

Peer review

This is a very interesting study, showing that pegylated IFN α-2b therapy can increase the frequency of specific CD8+ T lymphocytes in CHB patients. This may contribute to the better control of HBV replication and to the recovery of CHB patients, thus having a promise for therapeutic interventions. The experiments support the claim of the authors.

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BRIEF ARTICLE

Short-segment Barrett's esophagus and cardia intestinal metaplasia: A comparative analysis

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Abstract

AIM: To investigate the endoscopy and histology of short-segment Barrett's esophagus (SSBE) and cardia intestinal metaplasia (CIM), and their correlation with *Helicobacter pylori* (*H. pylori*) gastritis and gastroesophageal reflux disease (GERD).

METHODS: Biopsy specimens were taken from 32 SSBE patients and 41 CIM patients with normal appearance of the esophagogastric junction. Eight biopsy specimens from the lower esophagus, cardia, and gastric antrum were stained with hematoxylin/eosin, Alcian blue/periodic acid-Schiff, Alcian blue/high iron diamine and Gimenez dye. Results were graded independently by one pathologist.

RESULTS: The SSBE patients were younger than the CIM patients ($P < 0.01$). The incidence of dysplasia and incomplete intestinal metaplasia subtype was higher in SSBE patients than in CIM patients ($P < 0.01$). *H. pylori* infection was correlated with antral intestinal metaplasia ($P < 0.05$), but not with reflux symptomatic, endoscopic, or histological markers of GERD in CIM patients. SSBE was correlated with reflux symptomatic and endoscopic esophagitis ($P < 0.01$), but not with *H. pylori* infection and antral intestinal metaplasia.

CONCLUSION: Dysplasia risk is significantly greater in SSBE patients than in CIM patients. CIM is a manifestation of *H. pylori*-associated and multifocal atrophic gastritis, whereas SSBE may result from GERD.

Key words: Endoscopy; Barrett's esophagus; Cardia intestinal metaplasia; Esophagogastric junction; Gastroesophageal reflux disease

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INTRODUCTION

The incidence of adenocarcinoma in esophagus and gastroesophageal junction (GEJ) has increased in recent years in North America, Europe, Japan and China[1-3]. Barrett's esophagus (BE) is thought to be a premalignant condition of esophageal adenocarcinoma, accounting for most cases of adenocarcinoma of the GEJ. The reported prevalence of Barrett's-associated adenocarcinoma varies widely, with an average of 10%[4-7]. A meta-analysis[8] of 4120 patients

in China reported that BE is found in 2.44% of patients undergoing endoscopy for various symptoms of upper gastrointestinal tract diseases.

It was reported that the frequency of short-segment Barrett's esophagus (SSBE), < 3 cm in length, is increased and implicated as a risk factor for adenocarcinoma of the cardia[9-11]. Endoscopic diagnosis of this entity is difficult and always requires histological demonstration of specialized columnar epithelium (SCE). Since most endoscopists do not perform biopsies unless the columnar epithelium is seen to extend from the proximity to the GEJ. Short segments are frequently unrecognized. Spechler *et al*[12] have recently described the presence of intestinal metaplasia in certain normal-appearing GEJ. The relation of this condition to SSBE has not yet been investigated.

In this study, SSBE and cardia intestinal metaplasia (CIM) were compared and their correlation with *Helicobacter pylori* (*H. pylori*) gastritis and gastroesophageal reflux disease (GERD) was studied, which may contribute to the clinical diagnosis, treatment, prevention, and susceptibility forecast of BE.

MATERIALS AND METHODS

Patients

Tissue specimens used in this study were provided by The Sixth Hospital of Shanghai Jiaotong University, with the approval of the hospital and patients. Endoscopy was performed in a standardized manner by experienced endoscopists. Appearance of the squamocolumnar junction was carefully studied in a prograde view after insufflation of air and retroversion in the stomach. Thirty-two consecutive patients with endoscopically apparent SSBE (< 3 cm in length) included in the study (group A) were selected from The Outpatient Clinic of our hospital over a two-year period. Two endoscopic features of the squamocolumnar transition were considered indicative of SSBE: a straight and regular Z line (< 3 cm) displaced upwards in relation to the GEJ (circumferential type), and an irregular Z line with eccentric tongues of red mucosa extending above the GEJ (digital type). The severity of SSBE was measured according to the Prague C&M classification[13]. The specimens were stained with Alcian blue (pH 2.5).

Group B was consisted of 41 adult ambulatory consecutive patients who underwent upper endoscopy in our endoscopy unit and were considered by the endoscopist to have a normal-appearing GEJ. Patients with a history of cancer or prior gastric/esophageal surgery were excluded, as were those who were unable to give their informed consent, or who had any contraindication to endoscopic biopsies. CIM was defined based on the presence of barrel-shaped goblet cells in normal-appearing GEJ.

All patients included in this study were questioned about symptoms of GERD (heartburn, regurgitation, and odynophagia). Endoscopic signs of esophagitis were recorded and graded according to the Los Angeles classification[14].

Endoscopy and biopsy protocol

Biopsy specimens were taken from 32 patients with SSBE and 41 CIM patients with normal-appearing GEJ. Eight biopsy specimens, taken from the lower esophagus, cardia, and gastric antrum, were stained with hematoxylin/eosin, Alcian blue/periodic acid-Schiff (AB/PAS, pH 2.5), AB/high iron diamine (AB/HID) and Gimenez dye. Results were graded independently by one pathologist.

Table 1 Incidence of dysplasia in short-segment Barrett's esophagus and cardia intestinal metaplasia patients

Patients	*n*	Dysplasia	%
CIM	41	1	2.4
SSBE	32	4	12.5[b]

[b]$P < 0.01$ *vs* cardia intestinal metaplasia (CIM). Biopsy specimens taken from 41 CIM patients and 32 short-segment Barrett's esophagus (SSBE) patients were stained with hematoxylin and eosin. The incidence of dysplasia was calculated. The incidence of dysplasia was significantly higher in SSBE patients than in CIM patients (12.5% *vs* 2.4%, $P < 0.01$).

Histology

Formalin-fixed, paraffin-embedded biopsy samples were stained with hematoxylin/eosin. PAS/AB (pH 2.5) was used to show the presence of acid mucins. BE was diagnosed based on the presence of SCE, which was defined by the unequivocal demonstration of intestinal-type goblet cells.

Statistical analysis

Statistical analysis was performed using the χ^2 test.

RESULTS

Incidence of dysplasia in SSBE and CIM patients

The SSBE patients were younger than the CIM patients ($P < 0.01$). The incidence of dysplasia was higher in SSBE patients than in CIM patients ($P < 0.01$) (Table 1).

Incidence of incomplete intestinal metaplasia in SSBE and CIM patients

The incidence of incomplete intestinal metaplasia (IM) was significantly different between the two types of epithelium ($P < 0.01$ *vs* CIM) (Table 2).

Prevalence of GERD in SSBE and CIM patients

The prevalence of GERD symptoms was higher in SSBE patients than in CIM patients ($P < 0.01$), as was endoscopic and histological evidence of esophagitis (Table 3).

Correlation between H. pylori and antral IM in SSBE and CIM patients

The correlation between *H. pylori* infection and antral IM in SSBE and CIM patients is shown in Table 4.

DISCUSSION

Over the past two decades, the incidence of adenocarcinoma of the esophagus and gastric cardia has increased rapidly. BE is recognized as a precancerous lesion of esophageal adenocarcinoma in most cases of adenocarcinoma

Table 2 Incidence of incomplete intestinal metaplasia in short-segment Barrett's esophagus and cardia intestinal metaplasia patients

Patients	*n*	Incomplete IM	Complete IM	%
CIM	41	8	33	19.5
SSBE	32	21	11	65.6[b]
Total	73	29	44	39.7

[b]$P < 0.01$ *vs* cardia intestinal metaplasia (CIM). Eight biopsy specimens taken from the lower esophagus and cardia were stained with hematoxylin/eosin, Alcian blue/periodic acid-Schiff (pH 2.5), AB/high iron diamine or Gimenez dyes. The prevalence of incomplete intestinal metaplasia (IM) was significantly higher in short-segment Barrett's esophagus (SSBE) patients than in CIM patients (65.6% *vs* 19.5%, $P < 0.01$).

Table 3 Incidence of reflux symptomatic, endoscopic, or histological markers of gastroesophageal reflux disease in short-segment Barrett's esophagus and cardia intestinal metaplasia patients *n* (%)

Patients	*n*	Reflux symptoms	Endoscopic esophagitis	Histological features of reflux esophagitis
CIM	41	12 (29.3)	5 (12.2)	12 (29.3)
SSBE	32	26 (81.2)[b]	30 (93.8)[b]	31 (96.9)[b]

[b]$P < 0.01$ *vs* cardia intestinal metaplasia (CIM). All patients were questioned about symptoms of gastroesophageal reflux disease (GERD). Endoscopic signs of esophagitis were recorded and graded. All biopsy specimens were stained with hematoxylin and eosin. Alcian blue/periodic acid-Schiff (pH 2.5) was used to show the presence of acid mucins. The incidence of reflux symptomatic, endoscopic, or histological markers of GERD was higher in short-segment Barrett's esophagus (SSBE) patients than in CIM patients ($P < 0.01$).

Table 4 Relation between *Helicobacter pylori* infection and antral intestinal metaplasia in short-segment Barrett's esophagus and cardia intestinal metaplasia patients *n* (%)

Patients	*n*	Cardia *H. pylori* infection	Antral IM	Antral *H. pylori* infection
CIM	41	18 (43.9)	21 (51.2)	20 (48.8)
SSBE	32	5 (15.6)[a]	4 (12.5)[b]	7 (21.9)[a]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* cardia intestinal metaplasia (CIM). Eight biopsy specimens taken from the lower esophagus, cardia, and gastric antrum were stained with Alcian blue/periodic acid-Schiff (pH 2.5) and Gimenez dyes, respectively. The incidence of *Helicobacter pylori* (*H. pylori*) infection and antral intestinal metaplasia (IM) was lower in short-segment Barrett's esophagus (SSBE) patients than in CIM patients ([a]$P < 0.05$, [b]$P < 0.01$).

of the GEJ. Progression from metaplasia to dysplasia and adenocarcinoma is well documented[7]. Traditionally, BE is arbitrarily defined as a circumferential segment of columnar-lined epithelium (2 or 3 cm in length) in the lower esophagus. However, this macroscopic definition has been recently questioned, because it excludes shorter segments and "tongues of columnar-lined epithelium", which are frequently found in the distal esophagus, and endoscopic measurements can be imprecise. It has therefore been proposed that the diagnosis of BE should be reserved for patients with IM detected in biopsy specimens from the distal esophagus[15,16]. Recently, the presence of CIM in certain normal-appearing GEJ has been described[17-19]. Detection of IM in the distal esophagus as well as within the gastric cardia has been reported with an increasing frequency[15,16]. It was reported that the prevalence of BE and CIM is 2%-12% and 5%-23%, respectively, in patients undergoing routine upper gastrointestinal endoscopy[20,21]. Detection of IM in BE patients potentially commits the patients to regular surveillance endoscopy with biopsy. The incidence of adenocarcinoma in patients with BE is estimated to be 30-50 times greater than that in general populations, and is on the increase[6,7]. However, the exact incidence of cancer in patients with BE is unknown, and the role of CIM as a premalignant lesion is still unclear. The relation of this condition to BE has not yet been investigated. Whether CIM and IM originating from the esophageal mucosa have a common pathogenesis and identically associated risk factors remains unknown.

In the present study, the dysplasia risk was significantly higher in SSBE patients than in CIM patients (12.5% *vs* 2.4%). Sharma *et al*[15] also compared the incidence of dysplasia in 177 SSBE patients and 76 CIM patients. As in our study, the risk of dysplasia differed significantly between the two groups. Dysplasia was detected in 11.3% (20/177) of SSBE patients and in 1.3% (1/76) of CIM patients, indicating that dysplasia is two potentially different clinical processes. Future studies should separate SSBE from CIM to improve our understanding of the pathophysiology and malignant potential of each entity.

Although a few authors reported that the areas adjacent to CIM show normal foveolar epithelium, whereas those adjacent to BE contain pre-goblet cells that can be positively stained with Alcian blue[15,16]. Since these characteristics cannot be found in all biopsy specimens, it is not reliable to distinguish SSBE from CIM histologically. HID/AB staining has also been used to distinguish SSBE from CIM[17-19]. It was reported that IM at the GEJ (or ultra-short-segment BE) is more frequently found to express sulfomucins, which is defined as type Ⅲ IM and involves the surface glandular epithelium[11,17]. Liu *et al*[10] also found that the area covered by incomplete IM is significantly greater and the level of sulfomucins is obviously higher in the esophagus than in the stomach. In our study, the prevalence of type Ⅲ IM was significantly higher in SSBE patients than in CIM patients (65.6% *vs* 19.5%, $P < 0.01$). HID/AB staining can be used to distinguish SSBE from CIM initially, based on the different expressions of neutral mucins, sialomucins, and sulfomucins.

The incidence of reflux symptomatic, endoscopic, or histological markers of GERD was higher while that of *H. pylori* infection and antral IM was lower in SSBE patients than in CIM patients ($P < 0.05$). Since CIM is a manifestation of *H. pylori*-associated and multifocal atrophic gastritis, and SSBE can result from GERD, it is necessary to explore new and efficacious diagnostic methods to distinguish BE from CIM.

cDNA microarray methods have been used in the study of gene expression, DNA sequencing, novel genes and mutations, DNA polymorphism, drug screening, diagnosis of disease, and gene mapping, since they were reported by Schena *et al*[22] in 1995. We have previously

performed an analysis of three 4096 chips to investigate the difference in gene expression profiles between BE and CIM epithelium[23]. A total of 141 genes were screened that exhibited a differential expression in the three chips. A comparison between the two gene profiles showed that the gene expression patterns were different in BE and CIM epithelium, illustrating that detection of differences in gene expression between BE and CIM with gene chips is a new method for the diagnosis, treatment and prevention of BE. Future studies should separate SSBE from CIM to improve our understanding of the pathophysiology and malignant potential of such diseases.

COMMENTS

Background

The incidence of adenocarcinoma in the esophagus and gastroesophageal junction (GEJ) has increased in recent years. Barrett's esophagus (BE) is thought to be a premalignant condition. Recently, the presence of cardia intestinal metaplasia (CIM) in certain normal-appearing GEJ has been described. The relation between CIM and BE has not yet been investigated.

Research frontiers

Short-segment Barrett's esophagus (SSBE), < 3 cm in length, has been reported as a risk factor for adenocarcinoma of the cardia. Whether CIM and IM originating from the esophageal mucosa have a common pathogenesis still remains unknown. In this study, the authors demonstrated that CIM was a manifestation of *Helicobacter pylori* (*H. pylori*)-associated and multifocal atrophic gastritis, whereas SSBE could result from gastroesophageal reflux disease (GERD).

Applications

This study describing the he different characteristics of SSBE and CIM may contribute to the clinical diagnosis, treatment, prevention, and susceptibility forecast of BE.

Terminology

BE is thought to be a premalignant condition of esophageal adenocarcinoma, accounting for most cases of adenocarcinoma of the GEJ. BE is defined as IM detected in biopsy specimens from the distal esophagus. The extent of the Barrett segment is measured according to the Prague C&M classification.

Peer review

The authors examined the different characteristics of SSBE and CIM and revealed that CIM was a manifestation of *H. pylori*-associated and multifocal atrophic gastritis, whereas SSBE could result from GERD. The results are interesting and may contribute to the clinical diagnosis, treatment, prevention, and susceptibility forecast of BE.

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BRIEF ARTICLE

Increasing the frequency of CIK cells adoptive immunotherapy may decrease risk of death in gastric cancer patients

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Abstract

AIM: To analyze the correlation between cytokine-induced killer (CIK) cells adoptive immunotherapy and cancer-related death in gastric cancer patients.

METHODS: One hundred and fifty-six gastric cancer patients after operation at the Third Affiliated Hospital of Soochow University were enrolled in this study. Their clinical data including demographic characteristics, operation time, tumor size, pathological type and staging, tumor metastasis, outcome of chemotherapy or CIK cells adoptive immunotherapy, survival time or time of death were collected with a standard structured questionnaire. Kaplan-Meier method was used to estimate the median survival time, and the 2- and 5- year survival rates. Hazard risk (HR) and 95% confidence interval (95% CI) of CIK cells adoptive immunotherapy for gastric cancer were calculated using the two-stage time-dependent covariates Cox model.

RESULTS: The survival time of gastric cancer patients was longer after CIK cells adoptive immunotherapy than after chemotherapy (χ^2 = 10.907, P = 0.001). The median survival time of gastric cancer patients was also longer after CIK cells adoptive immunotherapy than after chemotherapy (49 mo *vs* 27 mo, P < 0.05). The 2- and 5-year survival rates of gastric cancer patients were significantly higher after CIK cells adoptive immunotherapy than after chemotherapy (73.5% *vs* 52.6%, 40.4% *vs* 23.9%, P < 0.05). A significant difference was observed in the survival curve for patients who received CIK cells adoptive immunotherapy (0, 1-10, 11-25, and over 25 frequencies) (χ^2 = 14.534, P = 0.002). The frequencies of CIK cells adoptive immunotherapy were significantly related with the decreasing risk of death in gastric cancer patients after adjustment for sex and age of the patients, tumor stage and relapse (HR = 0.54, 95% CI: 0.36-0.80) when the first stage Cox model was used to define the subjects who remained alive beyond 36 mo as survivors. However, no correlation was observed between the frequencies of death in CIK cells adoptive immunotherapy and the risk of gastric cancer patients (HR = 1.09, 95% CI: 0.63-0.89) when the second stage Cox model was used to define the subjects who survived for more than 36 mo as survivors.

CONCLUSION: The survival time of the gastric cancer

patients treated with chemotherapy combined with CIK cells adoptive immunotherapy is significantly longer than that of the patients treated with chemotherapy alone and increasing the frequency of CIK cells adoptive immunotherapy seems to benefit patients more.

Key words: Immunotherapy; Cytokine-induced killer cells; Gastric cancer; Survival analysis; Probability

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INTRODUCTION

Gastric cancer is one of the most common causes of cancer-related death in China[1]. Its incidence in Jiangsu Province of China is particularly high, and its mortality rate is much higher than the national average[2]. The early clinical detection rate of gastric cancer is less than 15%, and about 85% cases of gastric cancer are at the advanced stage when their gastric cancer is diagnosed[3]. Surgery is the standard treatment procedure for localized and resectable gastric cancer[4]. However, surgery alone does not improve the 5-year survival rate of local advanced gastric cancer patients[3]. Although standardized surgical resection and adjuvant therapeutic modalities are available for gastric cancer, the survival rate of advanced gastric cancer patients remains very low after operation[5]. About 60% of gastric cancer patients usually experience local recurrence and metastasis to other organs[6]. It has been demonstrated that local recurrence and distant metastasis constitute a major problem in the failure of cancer therapies[7]. Therefore, considerable efforts are needed to improve the current therapeutic modalities and to explore new therapies. In recent years, immune therapy has become the fourth important treatment modality for malignant tumors following surgery, radiotherapy and chemotherapy[8-10].

A number of adoptive immunotherapy with killer cells have been reported, including lymphokine-activated killer cells[11], tumor infiltrating lymphocytes[12], or anti-CD3 monoclonal antibody-induced killer cells[13]. However, their therapeutic efficacy is limited due to their low anti-tumor activities[14]. At present, cytokine-induced killer (CIK) cells are a new type of anti-tumor effector cells, which can proliferate rapidly *in vitro*, with a stronger anti-tumor activity and a broader target tumor spectrum than the reported anti-tumor effector cells[10,15]. Moreover, CIK cells can regulate and enhance immune function[16]. Studies have reported the level of tumor markers, change in cellular immune functions, exploration of molecule targets and a short-term efficacy in gastric cancer patients after chemotherapy plus CIK cells immunotherapy despite some side effects[17-19]. However, the relation between the frequencies of CIK cells immunotherapy and its clinical efficacy has not been examined. In the present study, data obtained from 156 gastric cancer patients in fitting multivariate Cox model showed that more frequencies of CIK cells immunotherapy could improve the survival rate of gastric cancer patients.

MATERIALS AND METHODS

Patients

One hundred and fifty-six primary gastric cancer patients after operation at the Third Affiliated Hospital of Soochow University (Jiangsu Province, China) were enrolled in this study. Those who did not meet the inclusion criteria, or had other tumors were excluded.

A standard questionnaire was designed to collect the data from the patients, including demographic characteristics, operation time, tumor size and location, pathological type and staging, tumor metastasis, outcome of chemotherapy or CIK cells immunotherapy. Meanwhile, time of relapse and death of the patients was recorded. Patients received 6 cycles of chemotherapy before CIK cells immunotherapy. Some patients underwent CIK cells immunotherapy due to cancer recurrence during chemotherapy. Recurrence of gastric cancer was defined when local, peritoneal or distant metastasis was detected at any site during chemotherapy[20]. The study was conducted according to the principles of the Declaration of Helsinki. All patients gave their informed consent prior to inclusion in the study. The study was approved by the Ethics Committee of the Third Affiliated Hospital of Soochow University.

Eighty-one patients (62 males, 19 females) at the age of 59.9 ± 10.5 years with a median age of 60.5 years who underwent chemotherapy alone served as chemotherapy group (group Ⅰ), and those (60 males at the age of 62.4 ± 10.8 years with a median age 60.5 years, 15 females at the age of 51.0 ± 10.7 years with a median age of 50 years) who received chemotherapy plus CIK cells immunotherapy served as treatment group (group Ⅱ) (Table 1).

Preparation of CIK cells and treatment

Peripheral blood mononuclear cells (PBMC) were collected with a COBE spectra blood cell separator (Gambro BCT, Inc., Lakewood, USA). Viability of PBMC was assessed by trypan blue exclusion. PBMC (2.0×10^6/mL) were plated onto 6-well dishes (Nunc, Denmark) and cultured with medium I containing RPMI 1640 in the presence of 1.0×10^6 U/L human interferon-γ (IFN-γ, Shanghai Fosun Pharma Co., China), 5.0×10^5 U/L recombinant human interleukin-2 (IL-2, Shangdong Quangang Pharmaceutical Co., China), 10% inactivated human serum, 25 mmol/L HEPES, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37℃. Monoclonal antibody (MAb) against CD3

Table 1 Distribution of demographic and clinical characteristics in two groups

Demographic and clinical features	*n*	Groups, *n* (%)		χ^2	*P*
		Group Ⅰ	Group Ⅱ		
Sex					
Men	122	62 (76.5)	60 (80)	0.273	0.601
Women	34	19 (23.5)	15 (20)		
Age (yr)					
≤ 45	14	7 (8.6)	7 (9.3)		
45 < age ≤ 60	71	36 (44.5)	35 (46.7)	0.047[a]	0.977
> 60	71	38 (46.9)	33 (44.0)		
Tumor site[b]					
Gastric cardia					
Yes	58	30 (37.0)	28 (37.3)	0.002	0.970
No	98	51 (63.0)	47 (62.7)		
Gastric body					
Yes	64	35 (43.2)	29 (38.7)	0.332	0.564
No	92	46 (56.8)	46 (61.3)		
Gastric antrum					
Yes	36	17 (21.0)	19 (25.3)	0.414	0.520
No	120	64 (79.0)	56 (74.7)		
Tumor size[c] (cm)					
< 5	76	43 (59.7)	33 (55.0)	0.299	0.585
≥ 5	56	29 (40.3)	27 (45.0)		
Histological type[c]					
Differentiated	52	28 (35.4)	24 (64.6)	0.002	0.962
Poorly-differentiated	94	51 (38.5)	43 (64.2)		
Invasion[c]					
Yes	112	55 (76.4)	57 (89.1)	3.745	0.053
No	24	17 (23.6)	7 (10.9)		
Lymph node metastasis[c]					
Yes	100	55 (76.4)	45 (70.3)	0.643	0.423
No	36	17 (23.6)	19 (29.7)		
Relapse					
Yes	98	58 (28.4)	40 (46.7)	5.566	0.018
No	58	23 (71.6)	35 (53.3)		
Pathological grade					
1	2	1 (1.3)	1 (1.3)		
2	27	18 (22.2)	9 (12.0)	2.976	0.395
3	92	44 (54.3)	48 (64.0)		
4	35	18 (22.2)	17 (22.7)		
Tumor stage					
Ⅰ	14	9 (11.1)	5 (6.7)		
Ⅱ	22	12 (14.8)	10 (13.3)	2.129[a]	0.546
Ⅲ	102	49 (60.5)	53 (70.7)		
Ⅳ	18	11 (13.6)	7 (9.3)		

[a]cmh2 χ^2-test; [b]Two cases of gastric cancer involving gastric cardia and body; [c]Missed cases.

(100 μg/L, Antibody Diagnostic Inc., USA) and IL-1α (1.0 × 10^5 U/L, Promega) were added after 24 h culture. Supernatant was aspirated and the cells were cultured in Medium Ⅱ in the absence of INF-γ after another 48 h culture. The cells were transferred to Kolle flasks (Nunc, Denmark) and cultured in the same medium after 1 wk. The medium was changed every 3 d. The cytotoxic activity was assayed as previously described[17,21]. Patients in group Ⅰ were treated with oxaliplatin (120 mg/m^2 D1, 5-Fu 400 mg/m^2 CIV 24 h D1-5, CF 200 mg/m^2 D1-5) as previously described[22] with the doses adjusted according to the toxicity. Patients in group Ⅱ received CIK cells immunotherapy as previously described[17] after 6 cycles of chemotherapy, with 1.0 × 10^9 CIK cells transfused into the patients within 1 h.

Statistical analysis

All data were loaded into the Epidata3.0 database with double-check, and analyzed with the SAS software package (version 9.13; SAS Institute, Cary, NC, USA). The data were expressed as mean ± SD. χ^2-test or cmh2 χ^2-test was used to compare the difference in balance between the concerned clinical indexes and to find the confounding factors between the two groups. Survival data were analyzed using Kaplan-Meier method and log-rank test to estimate the median survival time, 2- and 5-year survival rates, and to determine if the survival curves for the two groups were different.

When the frequency of CIK cells immunotherapy and the survival time were introduced into the Cox model, the interaction item was significantly associated with the death of gastric cancer patients (wald-χ^2 = 4.946, P = 0.0261). A two-stage time-dependent Cox model was established to precisely estimate the hazard risk (HR) and 95% confidence interval (95% CI) of the association between the frequency of CIK cells immunotherapy and the death of gastric cancer patients. Because the median survival time of gastric cancer patients was about 36 mo, 36 mo was used as the optimum cutoff point.

The first stage Cox model involved 154 patients with a survival time of over 36 mo who were defined as survivors. Otherwise, the survival status was the same as the original definition.

The second stage Cox model only involved 56 patients with a survival time longer than 36 mo, and their survival status was defined as the original definition.

Pearson correlation test was performed between Schoenfeld residual of the frequencies of CIK cells immunotherapy and the survival time of gastric cancer patients to determine whether the frequency of CIK cells immunotherapy is a time-dependent variable in the two Cox models[23].

RESULTS

Distribution of demographic and clinical characteristics in two groups

No statistical difference was found in sex and age of the patients, tumor site, histological type, invasion depth, lymph node metastasis, pathological grade, tumor size and stage between the two groups. However, the number of patients was significantly greater in group Ⅱ with recurrent disease than in group Ⅰ (46.7% *vs* 28.4%, χ^2 = 5.566, P = 0.018) (Table 1), suggesting that more patients with relapse should receive CIK cells immunotherapy.

Demographic and clinical characteristics of patients after CIK cells immunotherapy

No statistical difference was observed in sex and age of the patients, tumor site, histological type, invasion depth, lymph node metastasis, pathological grade or tumor size after CIK cells immunotherapy (0, 1-10, 11-25, and over 25 frequencies). However, a significant difference was found in cancer recurrence and stage after CIK cells immunotherapy (Table 2).

Table 2 Distribution of demographic and clinical characteristics in group Ⅱ

Demographic and clinical features	*n*	Frequency of CIK cells immunotherapy, *n* (%)				χ^2	*P*
		0	1-10	11-25	> 25		
Sex							
Men	122	62 (76.5)	39 (84.8)	13 (86.7)	8 (57.1)	5.573	0.134
Women	34	19 (23.5)	7 (15.2)	2 (13.3)	6 (42.9)		
Age (yr)							
⩽ 45	14	7 (8.6)	5 (10.9)	1 (6.6)	1 (7.1)	1.153	0.979
45 < age ⩽ 60	71	36 (44.4)	20 (43.5)	7 (46.7)	8 (57.1)		
> 60	71	38 (47.0)	21 (45.6)	7 (46.7)	5 (35.8)		
Tumor site[a]							
Gastric cardia							
Yes	58	30 (37.0)	17 (37.0)	5 (33.3)	6 (42.9)	0.290	0.962
No	98	51 (63.0)	29 (63.0)	10 (66.7)	8 (57.1)		
Gastric body							
Yes	64	35 (43.2)	19 (41.3)	5 (33.3)	5 (35.7)	0.691	0.875
No	92	46 (56.8)	27 (58.7)	10 (66.7)	9 (64.3)		
Gastric antrum							
Yes	36	17 (21.0)	10 (21.7)	4 (26.7)	5 (35.7)	1.614	0.656
No	120	64 (79.0)	36 (78.3)	11 (73.3)	9 (64.3)		
Tumor size[c] (cm)							
< 5	76	43 (59.7)	17 (45.9)	7 (58.3)	9 (81.8)	4.834	0.184
⩾ 5	56	29 (40.3)	20 (54.1)	5 (41.7)	2 (18.2)		
Histological type[c]							
Differentiated	52	28 (35.4)	12 (30.0)	6 (40.0)	6 (50.0)	1.760	0.624
Poorly-differentiated	94	51 (64.6)	28 (70.0)	9 (60.0)	6 (50.0)		
Invasion[c]							
Yes	112	55 (76.4)	38 (90.5)	10 (90.9)	9 (81.8)	4.226	0.238
No	24	17 (23.6)	4 (9.5)	1 (9.1)	2 (18.2)		
Lymph node metastasis[c]							
Yes	100	55 (76.4)	31 (75.6)	7 (58.3)	7 (63.6)	2.371	0.499
No	36	17 (23.6)	10 (24.4)	5 (41.7)	4 (36.4)		
Relapse							
Yes	98	58 (71.6)	31 (67.4)	8 (53.3)	1 (7.1)	15.633	0.0004
No	58	23 (28.4)	15 (32.6)	7 (46.7)	13 (92.9)		
Pathological grade							
1	2	1 (1.2)	1 (2.2)	0 (0.0)	0 (0.0)	2.976[b]	0.3953
2	27	18 (22.2)	8 (17.4)	0 (0.0)	1 (7.1)		
3	92	44 (54.3)	25 (54.4)	12 (80.0)	11 (78.6)		
4	35	18 (22.2)	12 (26.0)	3 (20.0)	2 (14.3)		
Tumor stage							
Ⅰ	14	9 (11.1)	3 (6.5)	0 (0.0)	2 (14.2)	13.66[b]	0.0386
Ⅱ	22	23 (28.4)	3 (6.5)	1 (6.7)	6 (42.9)		
Ⅲ	102	38 (46.9)	34 (73.9)	13 (86.7)	6 (42.9)		
Ⅳ	18	11 (13.6)	6 (13.1)	1 (6.6)	0 (0.0)		

[a]Tumor sites in some cases were repeated; [b]Because the theoretical value is less than 1, χ^2 was performed for patients who received cytokine-induced killer (CIK) cells immunotherapy at the frequencies of 11-25 and > 25, and for those who underwent CIK cells immunotherapy at the frequencies of 1-10, 11-25 and > 25; [c]Missed cases.

Survival time of patients in two groups

The survival time of gastric cancer patients in group Ⅱ was much longer than that of those in group Ⅰ (χ^2 = 10.907, P = 0.001, Figure 1). The median survival time of patients in group Ⅱ was also longer than that of those in group Ⅰ (49 mo *vs* 27 mo).

Two- and 5-year survival rates of patients in two groups

The 2- and 5-year survival rates of patients in group Ⅱ were significantly higher than those of patients in group Ⅰ (73.5% *vs* 52.6%, 40.4% *vs* 23.9%, P <0.05) (Table 3).

Survival time of patients after CIK cells immunotherapy

Because the CIK cells immunotherapy seemed effective against gastric cancer, whether the frequency of CIK cells immunotherapy affects its efficacy was determined. The survival curve was obviously higher for the patients after CIK cells immunotherapy plus chemotherapy than after chemotherapy alone. The survival time of gastric cancer patients was significantly longer after CIK cells immunotherapy than after chemotherapy (χ^2 = 14.534, P = 0.002, Figure 1).

Time-dependent Cox model analysis of CIK cells immunotherapy and prognosis of patients

The frequency of CIK cells immunotherapy was a strong time-dependent variable. A significant difference was observed in the survival time and the frequency of CIK cells immunotherapy between the two models (χ^2 = 27.990, P

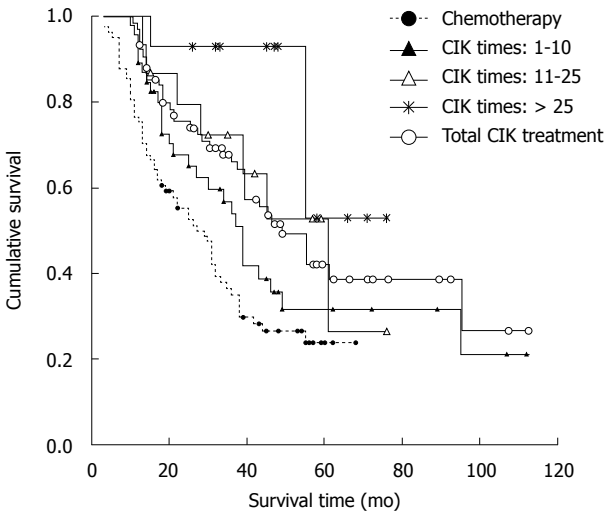

Figure 1 Survival rates of patients after cytokine-induced killer cells immunotherapy plus chemotherapy and chemotherapy alone. CIK: Cytokine-induced killer.

< 0.0001). However, it could not stratify the theoretical hypothesis of proportional hazard model. Hence, we tried to improve the analyzing results of the Cox model by dividing the patients into two stages with a relatively short survival time. Because of the relatively short interval time, the frequency of CIK cells immunotherapy may be a time-independent variable and can satisfy the assumption of proportional hazard model. The median survival time (36 mo) of the patients was used as the optimum cut-point.

In the first stage Cox model, the frequency of CIK cells immunotherapy was not a time-dependent variable, thus refusing the hypothesis of non proportional hazard model by Pearson correlation test between Schoenfeld residual of CIK cells immunotherapy frequency and survival time ($r = 0.04$, $F = 0.10$, $P = 0.751$). After the patients who remained alive beyond 36 mo after treatment were defined as survivors, the frequency of CIK cells immunotherapy was significantly associated with the decreasing risk of death in gastric cancer patients after adjustment for sex and age of the patients, tumor stage and relapse (HR = 0.54, 95% CI: 0.36-0.80) (Table 4).

In the second stage Cox model, the frequency of CIK cells immunotherapy was not a time-dependent variable ($r = 0.307$, $F = 1.98$, $P = 0.176$).When the second stage Cox model involving only 56 patients with a survival time of over 36 mo was fitted, no association was observed between the frequency of CIK cells immunotherapy and the survival time of gastric cancer patients (HR = 1.09, 95% CI: 0.63-0.89) (Table 5).

DISCUSSION

In China, gastric cancer patients are usually diagnosed at a relatively advanced stage with metastasis to other organs[24]. A number of treatment modalities are available for gastric cancer, such as surgical resection combined with chemotherapy[25,26], radiotherapy[27,28], thermotherapy[29,30] and/or traditional Chinese medicine[31,32]. However,

Table 3 Two- and five-year survival rates of gastric cancer patients in two groups

Groups	Survival rate (%)	95% CI	u-value	*P*
2-yr				
Chemotherapy	52.6	41.7-63.6	2.721	0.007
CIK treatment	73.5	63.3-83.7		
5-yr				
Chemotherapy	23.9	13.5-34.3	1.913	0.0526
CIK treatment	40.4	27.1-53.7		

CIK: Cytokine-induced killer; 95% CI: 95% confidence interval.

the 5-year survival rate of advanced gastric cancer patients is still very poor[33]. It has been shown that cellular immunotherapy can promote host anti-cancer immunity, thus prolonging the survival time of gastric cancer patients[34]. Treatment of gastric cancer with autologous CIK cells is one of the promising cellular immunotherapies[35].

The results of the present study demonstrate that CIK cells immunotherapy plus chemotherapy for gastric cancer has more potential benefits than chemotherapy alone. Therefore, the effect of adjuvant cellular therapies for gastric cancer has drawn more attention of oncologists. It has been shown that adjuvant radiotherapy and chemotherapy for gastric cancer after curative resection can improve the disease-free and overall survival time of gastric cancer patients[25,36]. Residual tumor cells after chemotherapy can be removed by the host immune system[37]. However, several cycles of chemotherapy can decrease the immune functions of gastric cancer patients[38], and have been suspected to be one of the reasons for the high relapse rate of gastric cancer after postoperative systemic chemotherapy. Immunotherapy can directly kill cancer cells and boost the immune responses against the tumor[39]. Therefore, immunotherapy should be beneficial for gastric cancer patients, and not conducive to the growth of tumor cells.

In the present study, CIK cells were obtained upon culturing PBMC in the presence of IFN-γ, IL-2, anti-CD3MAb, and IL-1α[13]. This method allows us to generate a large number of CIK cells. In addition, the anti-tumor cell activity of CIK cells is stronger than that of anti-tumor effector cells[16]. The effector cells in our culture are believed to be $CD3^+CD56^+$. CIK cells have a higher survival rate, proliferation capacity, and killing activity than their target cells[40,41], and can secrete a variety of cytokines, which further enhance the cytotoxicity of immune effector cells[42] and change the tumor microenvironment to favor cancer eradication. In addition, CIK cells can kill both autologous and allogeneic tumor cells[43,44], as well as multi-drug resistance cells and FasL-positive cells[45,46]. Accordingly, CIK cells immunotherapy combined with chemotherapy may have a synergistic effect.

Several studies[17,47-49] showed that CIK cells immunotherapy can significantly improve the immune functions of cancer patients, such as an increase in $CD3^+CD56^+$ level. However, the clinical data are not enough to demonstrate the effectiveness of CIK cells immunotherapy. The results of this retrospective study, based on the follow-up

Table 4 First stage time-dependent multivariate Cox model analysis of cytokine-induced killer cells immunotherapy at different frequencies and prognosis of the patients[1,2] (*n* = 154[3])

Variables[4]	β	$s_β$	Wald-χ^2	*P*-value	HR	95% CI
No. of CIK infusion	-0.620	0.200	9.592	0.002	0.54	0.36-0.80
Sex	0.294	0.298	0.969	0.325	1.34	0.75-2.41
Age	0.508	0.200	6.492	0.011	1.66	1.12-2.46
Tumor stages	0.739	0.202	13.377	0.0003	2.10	1.40-3.11
Relapse (yes or no)	3.363	0.719	21.848	< 0.0001	28.87	7.05-118.25

[1]All patients with survival time of longer than 36 mo (median value for total patients) were defined as the survivors; [2]Pearson correlation test between Schoenfeld residual of cytokine-induced killer (CIK) cells immunotherapy frequency and survival time (r = 0.01, F = 0.01, P = 0.936); [3]Missed patients; [4]Variable value definition in the Cox model (frequency of CIK cells immunotherapy: 0 = 0 time, 1 = 1-10 times, 2 = 11-25 times, 3 = more than 25 times; Sex: 1 = man, 2 = woman), age (0 = ≤ 45 yr, 1 = about 60 yr, 2 = over 60 yr), tumor stage (0 = stage Ⅰ, 1 = stage Ⅱ, 2 = stage Ⅲ, 3 = stage Ⅳ), relapse (1 = yes, 0 = no). HR: Hazard risk; 95% CI: 95% confidence interval.

Table 5 Second stage time-dependent multivariate Cox model analysis of cytokine-induced killer cells immunotherapy at different frequencies and prognosis of patients[1,2] (*n* = 56)

Variables[3]	β	$s_β$	Wald-χ^2	*P*-value	HR	95% CI
Number of CIK infusion	0.089	0.280	0.102	0.750	1.09	0.63-1.89
Sex	0.676	0.619	1.191	0.275	1.97	0.58-6.62
Age	-0.318	0.442	0.518	0.472	0.73	0.31-1.73
Tumor stages	-0.471	0.341	1.909	0.167	0.62	0.32-1.22
Relapse (yes or no)	2.203	0.558	15.582	< 0.0001	9.05	3.03-27.02

[1]This model only involving the patients with a survival time longer than 36 mo. The survival rate of each patient was similar; [2]Pearson correlation test between Schoenfeld residual of cytokine-induced killer (CIK) cells immunotherapy frequency and survival time (r = 0.307, F = 1.98, P = 0.176); [3]Variable value definition in the Cox model (frequency of CIK cells immunotherapy: 0 = 0 time, 1 = 1-10 times, 2 = 11-25 times, 3 = more than 25 times; Sex: 1 = man, 2 = woman), age (0 = ≤ 45 yr, 1 = about 60 yr, 2 = over 60 yr), tumor stage (0 = stage Ⅰ, 1 = stage Ⅱ, 2 = stage Ⅲ, 3 = stage Ⅳ), relapse (1 = yes, 0 = no). HR: Hazard risk; 95% CI: 95% confidence interval.

of 156 gastric cancer patients, show that the frequency of CIK cells immunotherapy can significantly prolong the survival time of gastric cancer patients.

However, the frequency of CIK cells immunotherapy may significantly decrease the risk of cancer-related death in gastric cancer patients. The common Cox model was not preferred in our analysis because the balance test showed that tumor relapse and stage were different in patients of the two groups. There are two reasons that support our conclusion. First, except for tumor relapse and stage, the balance test showed the following factors, including age and sex of the patients, tumor size, site and invasiveness, lymph node metastasis and pathological grade did not affect us to assign the gastric cancer patients into groups Ⅰ and Ⅱ. Second, due to the fact that the frequency of CIK cells immunotherapy was a time-dependent variable in the Cox model, a two-stage time-dependent Cox model adjustment was made for some confounding factors (tumor relapse and stage), thus the false results were avoided when the common Cox model was used to make our analysis more reliable.

After the postoperative adjuvant chemotherapy, most residual tumor cells sensitive to chemotherapy were removed. Chemotherapy may suppress the immune function and therefore immunotherapy is necessary for boosting immunity. It was reported that the number of $CD3^+$ cells and the $CD4^+/CD8^+$ ratio are significantly lower in most gastric cancer patients than in healthy controls after chemotherapy[17]. In the present study, the number of $CD3^+$ and $CD4^+$ cells was significantly increased, while the number of $CD8^+$ cells was declined and the $CD4^+/CD8^+$ ratio was increased after CIK cells immunotherapy, suggesting that CIK cells also have an immune modulating function in addition to their anti-tumor function[50]. Single CIK cells immunotherapy has an *in vivo* effect against gastric cancer for about one month[51]. In contrast, the number of $CD3^+$ cells and the $CD4^+/CD8^+$ ratio maintain at a high level after three cycles of CIK cells immunotherapy[17].

Our study has a few limitations. First, it was a retrospective cohort/observational study rather than a strictly-designed randomized trial. Since the patients were not assigned into CIK cells immunotherapy group and chemotherapy group, imbalance of certain clinical factors between the two groups could not be avoided. Second, there were some unknown potential reasons for choice of treatment regimens, which might affect our conclusion, despite the fact that the balance test was performed and some confounding factors were adjusted in the Cox model. Therefore, a randomized clinical trial is necessary to justify the benefit of CIK cells immunotherapy for gastric cancer. The benefit of radiochemotherapy and S1 chemotherapy for gastric cancers, established in a recent clinical trial[52], is important to determine whether CIK cells immunotherapy provides additional benefit when it is used in combination with radiotherapy and chemotherapy.

In conclusion, more frequencies of CIK cells are necessary for gastric cancer. The survival time of gastric cancer patients is significantly longer after chemotherapy plus CIK cells immunotherapy than after chemotherapy alone.

COMMENTS

Background

Gastric cancer is one of the most common causes of cancer-related death in China. Although standardized surgical resection and numerous adjuvant therapeutic modalities are available for gastric cancer, the postoperative survival rate of advanced stage cancer patients remains very low. In recent years, immune therapy for malignant tumors has become the fourth important tumor treatment modality following surgery, radiotherapy and chemotherapy. Cellular immunotherapy can promote host anti-cancer immunity, thus prolonging the survival time of gastric cancer patients. Treatment with autologous cytokine-induced killer (CIK) cells is one of the promising cellular immunotherapies.

Research frontiers

A number of adoptive cells immunotherapy have been reported, including using lymphokine activated killer cells, tumor infiltrating lymphocytes, and anti-CD3 monoclonal antibody-induced killer cells. However, their therapeutic efficacy is limited due to their low anti-tumor activities. CIK cells, a new type of anti-tumor effector cells, can proliferate rapidly *in vitro*, with a stronger anti-tumor activity and a broader spectrum of tumor targets than the reported anti-tumor effector cells. Moreover, CIK cells can regulate and enhance immune function.

Innovations and breakthroughs

CIK cells immunotherapy can decrease levels of tumor markers, change immune functions, and achieve a short-term efficacy against gastric cancer. However, the relation between the frequency of CIK cells immunotherapy and its clinical efficacy has not been examined. In the present study, data obtained from 156 gastric cancer patients were used in fitting multivariate Cox model, showing that more frequencies of CIK cells immunotherapy improve the survival rate of gastric cancer patients.

Applications

The survival time of gastric cancer patients was significantly longer after chemotherapy plus CIK cells immunotherapy than after chemotherapy alone, and more frequencies of CIK cells immunotherapy benefited gastric cancer patients more. This strategy can be applied in treatment of gastric cancer.

Terminology

CIK cells are cytokine-induced killer cells and a new type of anti-tumor effector cells, which can proliferate rapidly *in vitro* with a stronger anti-tumor activity and a broader spectrum of tumor targets than the reported anti-tumor effector cells. Moreover, CIK cells can regulate and enhance immune function.

Peer review

This study showed beneficial effect of CIK cells immunotherapy on gastric cancer, thus improving the 2- and 5-year survival rates of gastric cancer patients. The study is well designed and the data are believable.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the Management of Inflammatory Bowel Disease

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Instructions to authors

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

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

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Books

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

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