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EDITORIAL

Changing face of hepatic encephalopathy: Role of inflammation and oxidative stress

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Abstract

The face of hepatic encephalopathy (HE) is changing. This review explores how this neurocognitive disorder, which is associated with both acute and chronic liver injury, has grown to become a dynamic syndrome that spans a spectrum of neuropsychological impairment, from normal performance to coma. The central role of ammonia in the pathogenesis of HE remains incontrovertible. However, over the past 10 years, the HE community has begun to characterise the key roles of inflammation, infection, and oxidative/nitrosative stress in modulating the pathophysiological effects of ammonia on the astrocyte. This review explores the current thoughts and evidence base in this area and discusses the potential role of existing and novel therapies that might abrogate the oxidative and nitrosative stresses inflicted on the brain in patients with, or at risk of developing, HE.

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Key words: Hepatic encephalopathy; Ammonia; Inflammation; Oxidative stress; Astrocyte

INTRODUCTION

Hepatic encephalopathy (HE) is a neurocognitive disorder in which brain function is impaired and is associated with both acute and chronic liver dysfunction. HE occurs in the presence of liver injury or when the liver is bypassed in the presence of a portosystemic shunt. In acute liver failure, patients may develop cerebral oedema and increased intracranial pressure. However, recent studies suggest that intracranial hypertension is less frequent than previously described, complicating 25% of acute cases and only 9% of those with sub-acute liver failure[1]. In cirrhosis, it causes a range of neuropsychiatric and motor disturbances spanning a spectrum of abnormalities, which encompass short-term memory impairment, slowing of reaction time, poor concentration, psychomotor retardation, and sensory dysfunction, through to more clinically apparent neurological signs and symptoms. In its most severe form, patients can develop confusion, stupor, and coma[2]. However, abnormalities can be subtle and only become apparent on formal psychometric testing (minimal HE). Minimal HE is thought to be a disorder of executive functioning, primarily leading to impairments in selective atten-

tion, response inhibition, and working memory. This frequently impacts on quality of life[3] and specifically impairs navigation skills[4], which can be demonstrated utilising a driving simulator that correlates impairment with response inhibition and attention[5]. HE has generally been considered to be a reversible process following liver transplantation, although recent studies have suggested that this may not always be the case[6].

The "World Congress of Gastroenterology" in 2002 developed a set of consensus definitions, which has led to the classification of HE into three different types, A-C. (Table 1)[2]. In addition, the clinical presentation of HE was categorised into four main subtypes (Table 2). The heterogeneous nature of the presentation of HE has been the cause of great consternation, and has made the interpretation of comparative studies problematic. The staging of overt HE remains an imprecise art, which is often hampered by its fluctuant course. Thus, more objective methods using electroencephalographic techniques have been developed to assess HE. The effectiveness of using the bispectral index to grade and monitor the course of HE has high discriminative power in patients with both low and high grades of HE, and can be utilised as a simple and objective method of grading HE[7]. It has recently been suggested that we should consider HE as a spectrum of neurocognitive impairment in patients with cirrhosis; the spectrum spanning normal performance to coma[8].

Table 1 Classification of hepatic encephalopathy[2]

Type	Definition
A	Acute and hyperacute liver failure
B	Portosystemic bypass without intrinsic hepatocellular disease
C	Cirrhosis and portal hypertension with portosystemic shunts

A: Acute; B: Bypass; C: Cirrhosis.

Table 2 Clinical presentation of hepatic encephalopathy[2]

Encephalopathy	Definition
Acute	Acute liver dysfunction
Recurrent or episodic	Episodes of mental alteration in a patient with cirrhosis, even in the absence of a known precipitating factor
Persistent	Neurological deficit that persists despite the reversal of liver injury, such as following liver transplantation or the removal of a precipitating factor
Minimal (previously known as subclinical)	No evidence of overt encephalopathy, but subtle cognitive deficits might be detected with a neuropsychological function test battery

THE AMMONIA HYPOTHESIS

Ammonia was first thought to play a major role in the development of HE when studies by Hahn *et al*[9], Nencki *et al*[10] and Nencki *et al*[11] in the 1890s described the "meat intoxification syndrome". By diverting blood away from the liver utilising a surgical shunt from the portal vein into the vena cava of dogs, within 6 wk of the portocaval shunt being constructed, it was observed that the dogs developed symptoms such as aggression, irritability, and convulsions, similar to the symptoms exhibited by patients with cirrhosis and overt HE. The portocaval shunt allows blood to bypass the liver, resulting in a lack of urea metabolism, and arterial ammonia levels were found to be increased. When ammonium salts were administered to the dogs, they rapidly fell into a coma and died[9]. Ammonia was later confirmed as the main causative factor of the "meat intoxification syndrome" in portocaval shunted dogs in 1922[12]. The role of ammonia became increasingly recognised as being important when Gabuzda *et al*[13] and Phillips *et al*[14] attempted to treat patients with ascites with cation exchange resins that absorbed sodium but released ammonium ions, leading to the adverse effect of significant reversible neurological dysfunction, which was indistinguishable from the syndrome we now know as HE. Blood ammonia concentration was subsequently noted to be elevated in patients with liver disease and hepatic coma[15]; the highest values being found in those patients who were comatose[16].

Subsequently, other investigators have shown that ammonia plays a definitive role in the development of HE. Bessman *et al*[17] demonstrated a positive arteriovenous difference in ammonia levels in patients with cirrhosis, suggesting an uptake of free ammonia into the brain. More recently, Ehrlich *et al*[18] demonstrated that by constructing an end-to-side portocaval anastomosis in rats and injecting them with ammonium acetate, the rats demonstrated typical characteristics of HE, such as drowsiness, seizures, and coma in association with elevated blood and brain ammonia concentrations, compared to control rats. Lockwood *et al*[19] were then able to demonstrate the first evidence linking ammonia to HE in humans, using positron emission tomography (PET). A ^{13}N tracer demonstrated that the rate of uptake of ammonia in the brains of patients was greater in those with HE than without. It was postulated that an increased ammonia uptake in the brain was linked to an increased permeability of the blood-brain barrier to ammonia[20]. In acute liver failure, arterial ammonia concentrations of > 150 μmol/L predict a greater likelihood of dying from brain herniation[21], and intracranial hypertension develops in 55% of cases with an arterial ammonia concentration > 200 μmol/L[1]. In cirrhosis, there is no doubt that blood ammonia concentrations are elevated, but there is conflicting evidence regarding the relationship between ammonia concentration and HE severity. Moreover, it is not unusual in clinical practice to see patients with cirrhosis presenting with symptoms of overt HE who have normal or only mildly elevated arterial ammonia concentrations. Indeed, numerous studies have shown that a single test for blood ammonia concentration is a poor method for assessing HE[22]. Furthermore, Ong *et al*[23] studied the blood ammonia levels of patients with chronic liver disease and compared these to their mental states. In patients considered not to have any sign of HE, 60% had ammonia levels higher than normal, whereas there was a high proportion of those with grade 3 or 4

HE with normal or only mildly elevated blood ammonia levels. Whilst there is no denying the involvement of ammonia in the pathogenesis of HE, it seems that there might be other factors involved which are as, if not more, important.

THE ASTROCYTE IN HE

Astrocytes are a type of glial cell found within the central nervous system (CNS), which are involved in maintaining cells within the CNS, including providing nutrients for neurones. Astrocytes are particularly vulnerable to the effects of ammonia in the brain. One reason for this is that the enzyme glutamine synthetase is mainly located within astrocytes. Norenberg *et al*[24] found glutamine synthetase exclusively within astrocytes in rat brains, and none within neurones or other glial cells. It is also important to note that the end-processes of astrocytes surround the capillaries in the CNS. Theoretically, this would ensure that any toxin entering the brain, such as ammonia, is immediately metabolised, protecting other CNS cells from its damaging effects[25]. This theory was tested by Rao *et al*[26], who investigated the effects of ammonia exposure on purely neuronal cultures and co-cultures of neurons and astrocytes. The cultures containing neurons alone showed significant increases in cell death, apoptotic cells, degeneration of neuronal processes, and free radical levels. However, these changes were not detected in the co-cultures, indicating a protective function of astrocytes.

The blood brain barrier remains anatomically intact in HE[27]; however, PET studies have demonstrated an increased permeability-surface area to ammonia with increasing severity of disease[20].

HE in patients with chronic liver disease is characterised neuropathologically by Alzheimer type Ⅱ astrocytosis. This describes morphological changes to astrocytes, which include a large swollen nucleus, prominent nucleolus, and margination of the chromatin pattern. These neuropathological findings have been replicated in the brains of patients with congenital abnormalities of urea cycle enzymes[28], in experimental animal models[29,30], and astrocyte cultures exposed chronically to ammonia[31]. Therefore, it is likely that ammonia taken up into the brain interacts with astrocytes, eventually leading to these characteristic changes.

In acute liver failure, an increased brain ammonia concentration causes astrocyte swelling and patients develop cytotoxic brain oedema[32]. Kato *et al*[32] used electron microscopy to study the cells of patients who died of fulminant hepatic failure. They found brain oedema to be present, with pronounced swelling of astrocytes. Glutamine synthetase catalyses the conversion of ammonia and glutamate to glutamine. As a result, hyperammonemia can lead to excessive levels of glutamine within astrocytes, causing the cells to swell, and therefore explaining the oedema and intracranial hypertension seen with fulminant hepatic failure. Willard-Mack *et al*[33] used rats to investigate whether inducing an acute onset of hyperammonemia caused astrocytes to swell and if inhibiting the action of glutamine synthetase prevented these astrocytic changes. The study found that 8 h after inducing plasma hyperammonemia, changes in astrocyte morphology could be identified. These changes included an increased number of organelles, increased cytoplasmic volume, and an increased nuclear volume. They also found that inhibiting glutamine synthetase attenuated the enlargement of the nuclei and prevented the increase in astrocyte water content seen with hyperammonemia. The results of this study suggest that the production of glutamine by ammonia detoxification, results in water being drawn into astrocytes through osmotic pressure.

There might also be a potential role for vasogenic brain oedema in acute liver failure. This is believed to result from damage to the blood-brain barrier, leading to uncontrolled movement of plasma components and water to extracellular areas of the brain[34]. Consistent with this, animal studies have shown an increased permeability of the blood brain barrier to substances that are normally unable to cross it[35,36]. It has been suggested that perhaps in the early stages of HE, cytotoxic brain oedema predominates, and is enhanced in the later stages by vasogenic brain oedema following damage to the blood brain barrier[37]; however, the ability of mannitol to reduce intracranial hypertension in patients with fulminant hepatic failure indicates that the blood brain barrier remains largely intact[38].

The presence of low-grade astrocyte swelling has been further investigated in human patients with cirrhosis. Córdoba *et al*[39] used magnetic resonance spectroscopy and the magnetisation transfer ratio (a measure of free water in the brain) to assess cirrhotic patients before and after liver transplantation. The results showed a high level of free water in the brain before liver transplantation, which then reduced after transplantation. This correlated with changes in neuropsychological function, suggesting that brain oedema plays a direct role in the changes observed in HE. A further finding of the study was that brain glutamine levels also correlated with the changes in brain water and neuropsychological function, providing further evidence to the theory that hyperammonemia plays an important role in the pathophysiology of HE. Balata *et al*[40] showed that inducing hyperammonemia in patients with cirrhosis leads to an increase in brain glutamine, which results in an increase in brain water, and deterioration in neuropsychological function.

Interestingly, brain oedema and the consequent risk of intracranial hypertension are rarely complications of chronic liver failure, and are more often associated with fulminant hepatic failure. One possible suggestion for this is that in chronic liver disease, cells have more time to use compensatory mechanisms to adapt to the osmotic changes taking place[41].

Ammonia is directly toxic to the brain, and in acute liver failure causes disarray of inhibitory and excitatory neurotransmission[42], impairs brain energy metabolism[43-46], alters expression of several genes that code for important proteins involved in brain function[47,48], and impairs autoregulation of cerebral blood flow[49]. In patients with cirrhosis, there appears to be a shift in the balance between

inhibitory and excitatory neurotransmission towards a net increase in inhibitory neurotransmission.

THE CHANGING FACE OF HE

Although it is widely accepted that ammonia has a key role to play in the pathophysiology of HE, the clinical picture is not always so straightforward. Frequently, the arterial concentration of ammonia can be elevated in the absence of symptoms of HE, and the correlation between the severity of HE and ammonia concentration in patients with cirrhosis can be poor. The theory that several factors could contribute together to the clinical picture of HE was first suggested by Zieve *et al*[50] in 1974, who described the possible synergistic effects of several toxins, with ammonia. Since this first suggestion, it has become increasingly apparent that aspects of the inflammatory response (such as elevation of pro-inflammatory cytokines) in response to infection and/or systemic inflammation, and oxidative stress, participate in a synergistic relationship with ammonia in the pathogenesis of HE[51-53].

THE ROLE OF INFECTION AND INFLAMMATION IN HE

Acute liver failure

Studies in patients with acute liver failure have shown a more rapid progression to severe HE in those patients with evidence of a systemic inflammatory response, supporting a link between inflammation and HE[51]. In addition, in patients with acetaminophen-induced acute liver failure, infection and/or the resulting systemic inflammatory response were shown to be important factors contributing to an increase in the severity of HE[52]. Furthermore, in the advanced stages of HE in acute liver failure, the brain produces a number of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6[54,55]. This relationship is supported by evidence derived from therapeutic interventions, such as moderate hypothermia, that reduce cerebral oedema by reducing cerebral blood flow and inflammatory responses[56,57].

Cirrhosis

In patients with cirrhosis, there is mounting evidence for the role of inflammation in exacerbating the symptoms of HE, thus reinforcing the potential synergistic effects of ammonia and inflammation. Studies have shown this to be the case in patients with minimal HE, and across the whole spectrum of patients with varying degrees of overt HE (Westhaven grades 0-4)[53,58,59]. A recent study confirmed that the presence and severity of minimal HE in cirrhosis is independent of the severity of liver disease and plasma ammonia concentration, but markers of inflammation are significantly higher in those with minimal HE compared to those without[59]. In a further study, significant deterioration of neuropsychological test scores in patients with cirrhosis following induced hyperammonemia during the inflammatory state, but not after its resolution, suggested that inflammation might be important in modulating the cerebral effect of ammonia in liver disease, supporting an inflammatory hypothesis[53].

Synergy with ammonia

As inflammation, infection, and ammonia have been shown to be important in the pathogenesis of HE in cirrhosis, the question has to be raised as to whether infection and inflammation have a synergistic relationship with ammonia[60]. Marini and Broussard used mice with a deficiency in a critical urea cycle enzyme conferring chronic hyperammonemia, to demonstrate an increased sensitivity to inflammation. Furthermore, the hyperammonemic mice developed longer lasting and stronger cognitive defects when exposed to an inflammatory stimulus[61]. In a bile duct ligated (BDL) rat model, Jover *et al*[62] fed an ammonia-containing diet for 2 wk following ligation and compared animals sacrificed 7 d later to those fed a normal chow diet. Ammonia-fed BDL rats had increased cerebral ammonia and demonstrated the presence of type II Alzheimer astrocytosis analogous to patients with cirrhosis presenting with episodic HE. Both BDL groups had evidence of systemic inflammation, but the ammonia-fed BDL rats had increased brain glutamine, decreased brain myoinositol, and a significant increase in brain water compared to BDL controls, alluding to a potential synergistic relationship between ammonia and systemic inflammation. Wright *et al*[27] went on to explore the hypothesis that the inflammatory response induced by lipopolysaccharide (LPS) exacerbates brain oedema in BDL rats. LPS administration increased brain water in ammonia-fed, BDL, and sham-operated animals significantly, but this was associated with the progression to pre-coma only in the BDL animals. LPS induced cytotoxic brain swelling, but the anatomical integrity of the blood brain barrier was maintained. There was evidence of brain and systemic inflammation in BDL rats, which was significantly increased in LPS-treated animals. Nitrosation of proteins in the frontal cortex of BDL and LPS-treated animals was demonstrated. These data provide further evidence that in a background of cirrhosis and hyperammonemia, superimposed inflammation has an important role in the development of HE.

The ammonia-induced nitrosation of astrocytic proteins shown by Wright *et al*[27] has also been demonstrated in isolated astrocytes and astroglial tissue in brain sections of portocaval shunted rats[63]. However, ammonia alone cannot be responsible, because protein nitrosation was not demonstrated in ammonia fed sham-operated and ammonia-fed BDL rats in the absence of an inflammatory stimulus. Therefore, both ammonia and an additional inflammatory insult might need to be present for nitrosation of brain proteins to occur in animals with "subliminal" inflammation, such as that which has been observed in the BDL model[27]. This is further supported by recent work that demonstrated the presence of tyrosine nitration in astrocyte cultures in the presence of concentrations of TNF-α typically observed in patients with acute liver failure[64].

Inflammation and the brain

During an episode of infection, cytokines cannot directly cross the blood brain barrier and are unable to have a direct effect. Nevertheless, the peripheral immune system can still signal the brain to elicit a response during infection and inflammation through the expression of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6, both in the periphery and in the brain. Brain signalling may occur by direct transport of the cytokine across the blood brain barrier *via* an active transport mechanism, the interaction of the cytokine with circumventricular organs and activation of afferent neurons of the vagus nerve[65]. Endothelial cells, along with the astrocyte, are major constituents of the blood brain barrier. Endothelial cells are activated during infection, resulting in the release of various mediators into the brain. Activated microglial cells and astrocytes have the ability to produce a full repertoire of cytokines in response to inflammation and injury. One such cytokine is IL-1β, which has been shown *in vitro* to compromise the integrity of the blood brain barrier. This is mediated through the cyclo-oxygenase (COX) pathway within the endothelial cell[66]. In a portocaval shunted rat model that is more akin to a model of minimal HE, Cauli *et al*[67] demonstrated an improved learning ability following the administration of supra-therapeutic doses of the non-steroidal anti-inflammatory drug (NSAID), ibuprofen. This was accompanied by normalisation of COX and inducible NO activity within the cerebral cortex but interestingly also an increase in TNF-α. It is unclear however, how this NSAID specifically interacts with the glutamate-nitric oxide-cGMP pathway and how COX plays a role in the pathogenesis of minimal HE without identification of the specific COX isoform involved and in the absence of neuroanatomical, proteomic and genomic data. Nevertheless, the therapeutic use of NSAID in HE is not novel. Indomethacin (non-selective COX inhibitor) has been shown in patients with acute liver failure[68], and in a portocaval shunted rat model[69], to improve intracranial hypertension and cerebral oedema. Unfortunately, NSAID use is associated with a number of systemic complications, including cardiovascular/renal compromise and cellular prostaglandin metabolism, which impact greatly on not only astroglial function, but also on the development of organ dysfunction, particularly in the context of patients with longstanding liver disease.

TNF-α is released early during infection and can also influence the permeability of the blood brain barrier[70]. Moreover, an association between circulating TNF-α levels in patients with acute[71] and chronic liver failure[72] and the severity of HE, regardless of aetiology, has been recognised. Endothelial cells have receptors for IL-1β and TNF-α which can transduce signals which ultimately culminate in the intracerebral synthesis of NO and prostanoids[73]. Bémeur *et al*[74] investigated the effect of *IL-1β*, *TNF-α* and interferon-α (*IFN-α*) gene deletions on the onset of HE. Deletion of the *IFN-γ* gene had no effect on brain water levels or neuropsychiatric status. On the other hand, *IL-1β* and *TNF-α* gene deletions significantly delayed the onset of HE and brain oedema.

The relationship between the brain and inflammation is not one way. Molecular and neurophysiological studies during the past decade have suggested that pro-inflammatory responses are controlled by evolutionary neural circuits that operate reflexively[75,76]. The afferent arc of the reflex consists of nerves that sense injury and infection. This activates efferent neural circuits including the cholinergic anti-inflammatory pathway, which modulate immune responses and the progression of inflammatory disease. It might therefore be possible to target neural networks for the treatment of inflammation. This novel and fascinating body of work has recently been reviewed by Tracey[77].

Innate immune dysfunction

Innate immune dysfunction occurs in both acute and chronic liver failure, and up to 50% of admissions to hospital in patients with cirrhosis are likely to be related to the development of infection. In response to infection, the body initiates the innate immune response with phagocytic cells, such as monocytes and neutrophils. This response is particularly relevant to the liver, as the liver is the first organ to encounter bacteria or other toxins absorbed in the gut from the portal vein. Bacterial translocation of organisms from the gut in patients with cirrhosis and portal hypertension results in chronic endotoxemia. This culminates in a local milieu of pro-inflammatory cytokines/chemokines which can upregulate adhesion receptors and activate neutrophils[78]. There is significant literature on the immune response to infection in liver disease, which involves an important role of phagocytes and release of inflammatory cytokines. Patients with cirrhosis are functionally immunosuppressed and have impairment of several host defence mechanisms. The hemodynamic derangement of cirrhosis resembles that produced by endotoxin, and bacteremia can greatly exacerbate this state[79].

Neutrophils are a key component of the innate immune response. Ammonia has been shown to induce neutrophil dysfunction by inducing cell swelling, impaired phagocytosis, and increased oxidative burst in normal neutrophils *ex vivo*, in ammonia-fed rats and in patients with cirrhosis given an ammonia load[80]. Not only does this make patients potentially vulnerable to developing bacterial and fungal infections, but induces oxidative stress, and may ultimately culminate in a "sepsis-like" immune paralysis[81] and a reduction in monocyte HLA-DR expression[82].

OXIDATIVE STRESS

The evidence for the role of oxidative stress in the pathogenesis of HE is incontrovertible. Animal studies have shown significant reductions in the activities of glutathione peroxidase and superoxide dismutase enzymes, both in the liver and brain of rats exposed to ammonium acetate. Superoxide levels, in submitochondrial particles, were found to be elevated in ammonia-exposed rats[83] and lipid peroxidation has been shown to be increased, further demonstrating that hyperammonemia induces oxidative stress[84].

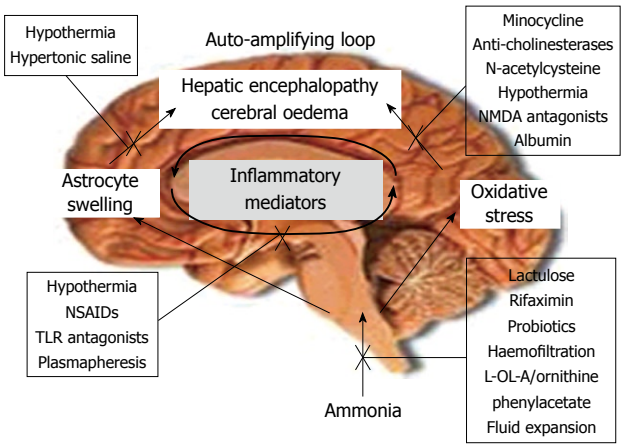

Figure 1 The "Two-hit" hypothesis. In a background of liver injury and hyperammonemia, a second "hit", such as an ammonia load following an upper gastrointestinal bleed, systemic inflammation/infection, or the development of hyponatremia can drive further astrocyte swelling, oxidative stress and lead to a rapid deterioration in neurocognitive function. The close relationship between astrocyte swelling and oxidative stress leads to an "auto-amplifying signalling loop". The sites of action of potential therapies are indicated on the Figure. L-OL-A: L-ornithine L aspartate; NMDA: N-methyl D-aspartate; NSAID: Non-steroidal anti-inflammatory; TLR: Toll-like receptor.

N-methyl D-aspartate (NMDA) receptors play a key role in the production of free radicals and an NMDA antagonist can prevent the calcium-mediated increase in oxidative stress[85]. *In vivo* excessive ammonia-induced NMDA receptor activation reduces antioxidant enzyme activity and results in increased production of superoxide anions[86]. It is, however, extremely difficult to differentiate whether it is oxidative stress that influences astrocyte swelling or whether astrocyte swelling itself induces oxidative stress through NMDA receptor and calcium-dependent mechanisms[87]. Either way, whether one considers that "the chicken came before the egg or vice versa", it would imply that the close relationship between astrocyte swelling and oxidative stress leads to an "auto-amplifying signalling loop" which promotes the development of HE[88] (Figure 1).

The production of reactive oxygen species (ROS) can arise in a number of different ways. Aside from ROS arising from neutrophil activation[80] and local and systemic inflammation/infection, ammonia and hypo-osmotic swelling-induced nitric oxide synthesis, the activation of NADPH oxidase[89], and mitochondrial glutamine uptake all generate ROS[90-92]. From these data we can propose a "two-hit hypothesis" in the pathogenesis of HE. Liver dysfunction leads invariably to hyperammonemia, which leads to astrocyte swelling, and in the longer term, structural changes to astrocytes (Alzheimer's type II astrocytosis). After this initial "hit", a second "hit", such as an ammonia load following an upper gastrointestinal bleed, systemic inflammation/infection, or the development of hyponatremia in a patient with cirrhosis can drive further astrocyte swelling, oxidative stress, and lead to a rapid deterioration in neuropsychological function (Figure 1 and Table 3).

Table 3 Factors precipitating hepatic encephalopathy[2]

Precipitating factors	Ammonia load e.g. upper gastrointestinal bleed or portocaval shunt
	Inflammation/oxidative stress
	Infection
	Dehydration
	Hyponatremia
	Sedative drugs e.g. benzodiazepines

Uptake of ammonia by astrocytes leads to the production of glutamine through the action of glutamine synthetase. Glutamine exposure in cultured astrocytes increases oxidative stress[91]. Mitochondrial glutamine uptake and subsequent cleavage of glutamine by phosphate-activated glutaminase elevates mitochondrial ammonia, which stimulates ROS production *via* induction of the mitochondrial permeability transition (MPT)[93]. However, cultured astrocytes exposed to ammonia produce ROS and begin swelling almost immediately, whereas MPT induction and glutamine accumulation occur thereafter.

Although astrocytes are relatively resistant to oxidative and nitrosative stress, neighbouring neurones are vulnerable to free radical attack. This can compromise brain energy metabolism and neurotransmission in patients with HE. Furthermore, ammonia, TNF-α, benzodiazepines, and hyponatremia can all trigger nitric oxide-dependent mobilisation of zinc which can augment GABAergic neurotransmission[94].

The mechanism through which free radical production is increased is currently not fully understood. One suggestion is based on findings that link an increase in calcium release to hyperammonemia. Rose *et al*[95] exposed cultured mice astrocytes to ammonium chloride. They observed a transient increase in the concentration of calcium ions from intracellular stores. The use of a calcium chelator (BAPTA) prevented the ammonia-induced production of free radicals[25]. Another possibility is that ROS are produced through activation of NMDA receptors[96].

One other area of research interest involves oxidation of RNA. It has been shown that in patients with Alzheimer's disease, there is significant RNA oxidation, which might result in impairments in protein synthesis and, consequently, cognitive function in patients[97]. Görg *et al*[98] reported the effects on cultured rat astrocytes and rat brain *in vivo* of ammonia exposure. Ammonia exposure was associated with a rapid, reversible oxidation of RNA (thought to involve NMDA receptor activation and calcium release). Consistent with this theory is the fact that some substrates required for learning and memory require protein synthesis[96]. Disruption of this protein synthesis *via* RNA oxidation might therefore interfere with cognitive function.

THERAPEUTIC STRATEGIES IN HE

To date, most therapeutic strategies in HE have been focused on lowering arterial concentrations of ammonia

and modulating inter-organ ammonia metabolism, but these remain largely ineffective. Treatments based on the hypothesis that the colon is the primary organ responsible for the generation of ammonia have ranged from dietary protein restriction, to the use of non-absorbable disaccharides, non-absorbable antibiotics, and colectomy[99]. However, Córdoba *et al*[100] showed that diets with normal protein content can be administered safely to patients with cirrhosis with episodic HE and that protein restriction does not have any beneficial effect for cirrhotic patients during an episode of HE and indeed, might even be detrimental in a patient with an underlying catabolic state.

It has been demonstrated that lactulose administered to patients with minimal HE in an unblinded open label study[101] might be of benefit and another open label randomised placebo controlled study in patients with a previous history of overt HE suggested that lactulose might delay the onset of a recurrent episode of HE[102]. However, in a recently published systematic review[103], which had very few high quality studies to base its findings on, lactulose was not found to have any impact on mortality in patients with cirrhosis presenting acutely with overt HE.

The use of non-absorbable antibiotics had been largely abandoned after concern that long-term administration of neomycin might lead to problems with nephrotoxicity and ototoxicity, and with metronidazole might lead to peripheral neuropathy. However, support for this strategy has been recently reinvigorated with the publication of the largest double blind placebo controlled study (n = 299) by Bass *et al*[104], which compared rifaximin (which has no known long term toxicity) favourably with placebo for the secondary prophylaxis of HE.

Benzodiazepine antagonists such as flumazenil also emerged as a potential therapy for HE patients. An analysis of six randomised controlled trials showed that 27% patients treated with flumazenil showed a clinical improvement, whilst 19% of treated patients showed an electroencephalographic improvement[105].

In the sickest cohorts, direct ammonia removal by hemofiltration in the intensive care unit is effective, but unfortunately by this stage multiorgan dysfunction and bacteremia might have superseded. Likewise, albumin dialysis in patients with acute-on-chronic liver failure improves HE grade[106], but the improvement is independent of changes in ammonia or cytokines[107] and remains controversial[108].

To address the issue of inter-organ ammonia metabolism, recent studies in patients with cirrhosis have shown that other than the gut, kidneys and muscle might be important targets[99]. Volume expansion produces significant increases in renal ammonia excretion resulting in a reduction in plasma ammonia concentration. This was shown to improve mental state, supporting the notion that the kidneys can be manipulated favourably[109]. During the hyperammonemic state, muscle detoxifies ammonia through conversion to glutamine[110,111]. L-ornithine L-aspartate (LOLA), which is a mixture of two amino acids, provides intermediates that increase glutamate availability for synthesis of glutamine and illustrates the concept that muscle can detoxify ammonia. Administration to animals with acute liver failure resulted in reduced brain water[112], but a recent study in patients with acute liver failure did not have any impact on brain dysfunction or survival[113]. When given to patients with cirrhosis and HE, administration of LOLA resulted in an improvement in HE compared with placebo-treated controls[114], although a recent meta-analysis concluded that it had little effect in patients with minimal HE[115]. Jalan *et al*[116] have hypothesised that this inefficacy might result from an accumulation of glutamine resulting in a rebound rise in circulating ammonia. By utilising a strategy which enables the excretion of glutamine, Davies *et al*[117] have demonstrated a synergy between L-ornithine and phenylacetate in reducing arterial ammonia in BDL rats.

However, in this review we have already convincingly demonstrated that ammonia, although central in the development of HE, is not solely responsible for its development. Infection/inflammation and oxidative stress are key determinants and indeed act synergistically with ammonia. Although ammonia could potentially be responsible for the development of neutrophil dysfunction, a patient with cirrhosis presents independently as a model of chronic endotoxemia that has direct implications on the innate and adaptive immune systems. We must therefore also look to therapies that directly or indirectly target the proinflammatory milieu.

Potential therapeutic strategies might include NMDA antagonists[84], leukodepletion[118], antagonism of pro-inflammatory cytokines[119], antioxidants [N-acetylcysteine (NAC)[120] and albumin[107,121]], anti-inflammatories (COX inhibitors[67] and minocycline[122,123]), probiotics[124] and hypothermia[125]. Excitement surrounds the prospect of small molecules that modulate toll-like receptor (TLR)-4 signalling, which can potentially down regulate neutrophil activation and other cellular responses. Early data indicate that TLR-4 antagonists can reduce LPS-stimulated cytokine release in healthy volunteers and results from phase 3 clinical trials are awaited. Inhibition of TLR-2, 4, and 9 prevented the increase in neutrophil oxidative burst induced from plasma from patients with alcoholic hepatitis. Furthermore, albumin, an endotoxin scavenger, prevented the deleterious effect of patients' plasma on neutrophil phagocytosis, spontaneous oxidative burst, and TLR expression[121]. This might also explain the beneficial role of albumin dialysis on HE[107,126].

When administered early after an overdose of acetaminophen, intravenous NAC prevents hepatic necrosis by replenishing stores of glutathione[127]. In patients with acute liver failure secondary to an overdose of acetaminophen, and in patients with acute liver failure secondary to other causes, NAC has been shown to increase oxygen delivery and consumption associated with increases in mean arterial pressure, cardiac index[128], and cerebral perfusion pressure[120]. These beneficial hemodynamic effects have been shown to be mediated by enhanced activity of the nitric oxide/soluble cGMP system[129] and suggest that NAC could have a beneficial role in the treatment of patients with cirrhosis who have developed overt HE.

As the role of central pro-inflammatory mechanisms are believed to be important in the pathogenesis of HE, then another novel therapeutic candidate drug to be considered is minocycline, which has been shown in two very recent studies by Jiang *et al*[122,123] to have anti-inflammatory effects in rats with acute liver failure. Minocyline treatment prevented both microglial activation [CD11b/c (OX-42) expression on immunohistochemistry] as well as the upregulation of IL-1β, IL-6, TNF-α, heme-oxygenase-1, eNOS, iNOS mRNA and protein expression with a concomitant attenuation of the progression of HE and brain edema, and at least in part, by reduction of oxidative/nitrosative stress. Thus, minocycline might also have promise in patients with acute and chronic liver failure cirrhosis and HE, and could be taken forward into randomised placebo controlled trials.

Modulation of intestinal microbiota is an emerging strategy to reduce the bacterial translocation of LPS and other bacterial activators of TLRs. Probiotics have been shown to reduce bacterial translocation and were shown to improve liver function and prevent the development of infection and HE in patients with cirrhosis[124]. Furthermore, probiotics have been shown to restore neutrophil phagocytic capacity in patients with alcoholic cirrhosis, possibly by reducing endogenous levels of IL-10 and TLR-4 expression[130].

Recent studies show that hypothermia is efficacious in patients with uncontrolled intracranial hypertension that are undergoing liver transplantation[56,125]. Hypothermia displays many beneficial effects on brain water and intracranial hypertension relating to decreased brain ammonia, cerebral blood flow, mediators of inflammation, and oxidative stress[131]. The sites of action of potential therapies for HE is shown in Figure 1.

CONCLUSION

HE is a dynamic neuropsychological spectral disorder that develops after liver injury. The pathophysiological mechanisms behind the development of HE are still not fully understood, but ammonia and the downstream consequences of ammonia uptake by astrocytes remain fundamental to the process. Ammonia not only leads to astrocyte swelling, but also alters neurotransmission, mitochondrial function, and induces oxidative stress. Astrocyte swelling and oxidative stress are closely related and result in "an auto-amplifying" loop. The presence of local and systemic inflammation and the release of ROS further exacerbate the cerebral effects of ammonia. Anti-inflammatory and anti-oxidative strategies may abrogate these effects and offer real treatment options to patients with HE in the future.

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EDITORIAL

R0 resection in the treatment of gastric cancer: Room for improvement

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Abstract

Gastric carcinoma is one of the most frequent malignancies in the world and its clinical behavior especially depends on the metastatic potential of the tumor. In particular, lymphatic metastasis is one of the main predictors of tumor recurrence and survival, and current pathological staging systems reflect the concept that lymphatic spread is the most relevant prognostic factor in patients undergoing curative resection. This is compounded by the observation that two-thirds of gastric cancer in the Western world presents at an advanced stage, with lymph node metastasis at diagnosis. All current therapeutic efforts in gastric cancer are directed toward individualization of therapeutic protocols, tailoring the extent of resection and the administration of preoperative and postoperative treatment. The goals of all these strategies are to improve prognosis towards the achievement of a curative resection (R0 resection) with minimal morbidity and mortality, and better postoperative quality of life.

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Key words: Gastric cancer; R0 resection; Total gastrectomy; Lymph node dissection; Adjuvant therapy; Preoperative therapy

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INTRODUCTION

Despite an incidence rate that has steadily declined over the past few decades, gastric carcinoma is one of the most frequent malignancies worldwide. An estimated 934 000 new cases are diagnosed each year, with the highest incidence rate in Northeast Asia, intermediate incidence rates in Europe and South America, and the lowest incidence rates in North America, Africa, South Asia, and Oceania[1,2].

Early dissemination of the disease through the lymphatic system, blood, and peritoneum has limited optimal surgery as a cure, except in patients with early-stage cancers. In Japan and Korea, the introduction of screening for gastric cancer has been shown to improve early detection, and almost half of newly diagnosed patients are detected at an early stage[3-6]. Due to the lower disease incidence rate, this strategy has not been deemed cost-

effective in Europe or North America. Consequently, two-thirds of gastric cancers in the Western world present at an advanced stage, with lymph node metastasis at the time of diagnosis[7].

The mainstay of treatment is radical surgery, but even with optimal surgical resection, the prognosis remains dismal in Western countries. Numerous attempts have been undertaken to improve clinical outcomes. To date, most therapeutic efforts are directed toward an individualization of therapeutic protocols, tailoring the extent of surgery and integrating it with the administration of preoperative and/or postoperative treatment. The goal of such strategies is to improve prognosis towards the achievement of a curative resection with minimal morbidity and mortality and better postoperative quality of life.

R0 RESECTION: DEFINITIONS

Curative resection refers to the absence of tumor after surgical treatment, and in the Western world, it meets the R0 resection definition provided by Hermanek *et al*[8] more than 15 years ago. R0 resection indicates a microscopically margin-negative resection, in which no gross or microscopic tumor remains in the primary tumor bed. R1 resection indicates the removal of all macroscopic disease, but microscopic margins are positive for tumor. R2 indicates gross residual disease with gross residual tumor that was not resected (primary tumor, regional nodes, and macroscopic margin involvement).

If this definition holds, R0 resection should represent a surgical cure, with a high survival rate and low recurrence. Considering the low survival rate after R0 surgical treatment in the Western case-mix, it is clear that the R0 definition needs to be revised, especially in locally advanced cases[9-11]. It is likely that there is a tendency to misclassify a number of cases as R0 resection, which inexorably will recur, which suggests that a curative treatment was not actually achieved.

The reason that the definition of Hermaneck is not in accordance with this scenario may be because it is mainly concerned with the primary tumor site, and not examining in detail the three pathways of tumor dissemination: portal blood stream to the liver, peritoneal surfaces and lymphatic dissemination. With these methods of dissemination, it is often beyond the surgeon's ability to achieve loco-regional control of the cancer. It may be difficult or impossible for the surgeon to reduce the incidence of metastases to the liver, as well as to contain the peritoneal seeding of cancer cells, or the removal of all extra-regional metastatic lymph nodes.

In the eastern world, Japanese guidelines have given a different definition to the curative gastric resection based on both surgical and histopathological details[12]. Resection A: no residual disease, with a high cure probability. It implies resections satisfying all of the following conditions: tumor without serosal invasion; N0 treated by D1, D2, or D3 lymph node dissections, or tumor with first-level lymph node treated by D2 or D3 resection; no distant, peritoneal or liver metastases, negative cytological examination of peritoneal fluid and proximal and distal margins > 10 mm. Resection B: no histopathologic residual disease but not fulfilling criteria for resection A. Resection C: definite residual disease.

These strict criteria emphasize that once the tumor penetrates the serosa or invades adjacent organs, it begins to spread by routes other than the regional lymphatic system. Specifically, tumor metastasis can occur through the peritoneum, extra-regional lymph nodes and the portal-hepatic blood, which consequently diminishes the probability of a cure. Such a definition would imply that more than two-thirds of patients are considered non-curatively treated by surgery in the Western world, which underestimates the role of surgery at these stages.

Today, both definitions seem inadequate: they merely indicate the absence or presence of residual tumor cells in the tumor bed after surgical treatments or provide an estimation of the probability of cure with surgery. In reality, the surgeons must consider themselves responsible not only for resection of the large mass of the primary cancer and overt lymph node metastases in the tumor bed, but also for dealing with microscopic and distant residual disease.

R0 RESECTION AND PREOPERATIVE IMAGING: WHAT CAN WE ANTICIPATE?

Although surgical pathology provides the most accurate information on tumor extent, clinical preoperative staging is crucial to select the appropriate treatment strategy. Today, clinical staging has been improved by technical enhancement in endoscopic ultrasound (EUS), computed tomography (CT), positron emission tomography (PET), combined PET-CT scan, magnetic resonance imaging (MRI) and laparoscopic staging. Presently, EUS and CT are widely used for preoperative staging[13].

Although the accuracy of T staging has been much improved for EUS (current range: 78%-92%)[14-20] and CT (current range: 69%-89%)[17,21-27], N staging accuracy is still poor (63%-78% in EUS[14-20], 51%-78% in CT[17,21-27]). MRI has had limited use in the staging of gastric cancer, primarily as a result of difficulties with motion artifacts, cost, time required for examination, and lack of an appropriate oral contrast agent[28,29]. However, in recent studies, overall T staging accuracy has been reported to be between 71.4% and 82%, which is similar to CT[29]. In N staging, several studies have shown that the accuracy of MRI nodal staging is inferior to CT staging with both techniques tending to understage nodal status[28,29]. Moreover, MRI has showed a greater sensitivity than CT in detecting liver, bone, and peritoneal dissemination[29].

Generally, PET is not routinely performed in the clinical staging of gastric cancer. From clinical studies focusing on PET, it is concluded that, for N staging, PET has a significantly higher specificity (92%) but lower sensibility (56%) compared to CT in the detection of local lymph node involvement[30-32]. Recent reports have confirmed the limited role of PET in the preoperative staging of gastric cancer, but it must be pointed out

that combined PET-CT can significantly improve overall staging accuracy compared to PET and CT alone[33,34].

Due to the inaccuracy of CT for the detection of ≤ 5 mm macrometastases on the peritoneal surface or liver, staging laparoscopy is recommended as the next step in the evaluation of patients with locoregional disease. Staging laparoscopy can detect metastatic disease or modified preoperative therapeutic strategy in 23%-54% of patients, thus confirming its crucial role in staging gastric carcinoma[35-37]. Moreover, there is some evidence that laparoscopy permits a more accurate staging of extraserosal tumors, whereas EUS might sometimes lead to misinterpretation of T3 invasion, when edema distorts the interface between the stomach and adjacent tissues[18,38,39].

In addition, staging laparoscopy facilitates cytological examination of abdominal lavage fluid. Cytology of peritoneal fluid or lavage may reveal the presence of free intraperitoneal gastric cancer cells, which identifies patients with an otherwise occult microscopic carcinomatosis. Recent evidence has suggested that patients with positive findings on peritoneal cytology have a poor prognosis, similar to that of patients with macroscopic stage Ⅳ disease[40].

SURGICAL DEBATES OF R0 RESECTION

R0 resection: Total vs subtotal gastrectomy, what else?

Some issues about the extent of gastric resection seem to have been settled. Total gastrectomy should be avoided if adequate free resection margins can be obtained with subtotal gastrectomy: a gross surgical margin of at least 5 cm for the intestinal type or 8-10 cm for the diffuse type[41-44]. Many authors agree on the necessity of total gastrectomy if the cancer encroaches on an imaginary line between the angula incisura of the lesser curvature and the "bare" area on the greater curvature between the gastroepiploic vessels and the short gastric vessels[44]. This is because the lymph drainage from such a tumor feeds into the splenic hilum and flows along the splenic artery, as well as passing proximally and distally.

Proximal tumors and tumors of the gastroesophageal junction (GEJ) deserve different considerations. These tumors are traditionally classified according to the Siewert classification system, which takes into account the center of the tumor and the variable involvement of the esophagus and stomach: type Ⅰ, esophageal adenocarcinoma of the distal esophagus, with the center located between 1 and 5 cm above the GEJ; type Ⅱ, true adenocarcinoma of the cardia located within 1 cm above and 2 cm below the GEJ; and type Ⅲ, subcardial adenocarcinoma located between 2 and 5 cm below the GEJ. Surgical treatment of these tumors usually requires an extended total gastrectomy with resection of variable portions of the distal esophagus. The extent of resection of the distal esophagus depends on the extent of the tumor spread[45].

Generally, patients with type Ⅰ tumors are best treated by esophagectomy with gastric pull-up to the neck or by esophagogastrectomy (transthoracic or transhiatal). Type Ⅱ and Ⅲ tumors can be resected by gastrectomy with frozen-section-guided resection of the distal esophagus (transhiatally extended gastrectomy)[46]. Although total gastrectomy has been the procedure of choice in these tumors, some authors have advocated proximal gastrectomy as a surgical option, and in a retrospective study conducted by the Memorial Sloan Kettering Cancer Center, proximal gastrectomy has been reported to have similar mortality rate, hospital stay, and recurrence and survival rates[47]. Even if the R0 resection rate does not differ between groups, other authors have reported poor functional and quality of life results in patients undergoing proximal resection[48-50]. Although it is difficult to make definitive conclusions in the absence of a prospective randomized trial, it does appear that total gastrectomy remains the procedure of choice in these patients.

R0 resection: The "circumferential/lateral" margin

The progression of the cancer through the stomach wall to the adjacent structures makes one aware of the concept of circumferential/lateral margins and provides the rationale for conservative and extended surgery.

If diagnosed at an early stage, it may be possible to obtain a margin-negative resection without traditional gastrectomy (subtotal, proximal or total gastrectomy). When margin-free resection is warranted, the only limiting factor is the risk of lymph node metastasis. For patients with a well- to moderately well-differentiated tumor of less than 2 cm in size, with no submucosal invasion or lymphangioinvasion, local excision by endoscopic mucosal resection (EMR) has been the preferred treatment in Japan for the past 15 years, since the risk of lymph node metastases is thought to be very low[51].

Although a prospective randomized trial is lacking in the literature, results of a systematic review of cohort studies have shown that EMR has favorable disease-specific survival, incidence of local recurrence and complications, compared with surgery[52,53]. Endoscopic submucosal dissection (ESD) is a newly developed technique that can remove large tumors in one piece. In a comparison with EMR, resection that removes tumors in one piece was more frequent in an ESD group and resulted in a better 3-year recurrence-free rate, despite a higher complication rate[54,55].

Currently, indications for ESD, according to Japanese guidelines, are only for well-differentiated intramucosal (T1a) tumors. However, a large-scale study analyzing lymph node metastasis of early cancer has expanded the criteria for endoscopic treatment of early gastric cancer, which is based on tumor characteristics with a very low risk of lymph node metastasis[56]. This study showed that patients with intramucosally or submucosally well-differentiated tumors of less than 3 cm and poorly intramucosally differentiated tumors of less than 2 cm have a very low risk of lymph node metastasis.

The results of both the United Kingdom Medical Research Council and the Dutch trials, along with more recently randomized controlled trials, large retrospective series and meta-analysis[57-63] have reported a significantly worse prognosis, higher mortality, higher complication

rate, and longer hospital stay in patients who have undergone gastrectomy with prophylactic splenectomy or pancreaticosplenectomy.

Theoretically, in patients with T4 gastric adenocarcinoma, extended resection is required to improve the R0 resection rate. With careful patient selection, gastrectomy with additional organ resection can be done with acceptable morbidity and low mortality. Improvements in preoperative evaluation to confirm T3 and T4 disease are needed because postoperative histopathological examination has revealed that multi-organ resections are often performed for pT3 tumors, with a relatively small proportion of pT4 tumors[64,65]. Independent factors of a worse prognosis, such as N3 tumors and large diameter tumors (> 10 cm), have to be excluded before performing extended resection[66,67]. Based upon these issues, the cautious clinical behavior is to reconsider any clinically defined T4 tumor on a case by case basis before planning extended multi-organ resection.

R0-resection: When can the lymph node dissection be considered margin-negative?

The extent of lymphadenectomy continues to represent the main area for surgical research in gastric cancer, and the surgical strategy of choice is still a matter for debate. Lymphatic metastasis is one of the main predictors of tumor recurrence, and survival and current pathological staging systems reflect the concept that lymphatic spread is the most relevant prognostic factor in patients resected with curative intent[68-73]. Recurrence rates attributed to residual lymph node metastasis around the celiac artery have led to the concept that complete clearance of the metastatic lymph nodes by extended dissection (D2) may prolong survival. In Japan, where gastric cancer is far more common than in Western countries, a standardized lymph node dissection has been developed over the past 40 years and is used nationwide with therapeutic benefit and long-term survival rates of $\geqslant$ 60% after 5 years. Retrospective studies from Japan, and later from Korea[74], involving more than 10 000 patients, have suggested that extended lymph node dissection combined with gastrectomy increases 5-year survival rate from 50% to 62%, compared to a 5-year survival rate of 15%-30%, as a result of limited resections in the United States[75-79].

The importance of adequate lymph node dissection as part of a potentially curative resection has led to the development and publication of "The General Rules for the Gastric Cancer Study in Surgery and Pathology", which was definitively published in English in 1996[12]. Several Western reports have confirmed that extended lymphadenectomy, similar to that recommended in the General Rules, can be safely performed with improvements in survival[80-85].

In the Western world, the challenge has been to show whether these results could be generalized for unselected patients. To date, four prospective randomized trials of Japanese-defined D1 *vs* D2 lymph node dissection and two meta-analysis studies have been conducted[86-92].

All of these studies have documented limited survival benefits with unacceptable morbidity and mortality that is probably associated with pancreaticosplenectomy, low case volume, and a lack of specialist training[93,94]. Moreover, some authors have suggested that extended lymph node dissection combined with rigorous pathological evaluation results in improved staging rather than therapeutic benefit. Through accurate staging, patients with advanced stage cancer are well categorized, and any comparisons with series of non-standardized lymph node dissection, or under-staged patients, are therefore inaccurate. These results have made many Western surgeons reluctant to perform extended lymph node dissection routinely in an effort to obtain better regional disease control, and possibly, some survival advantage. Nevertheless, there is some evidence that extended lymph node dissection may offer a definite chance for a cure in a subset of patients with pN2 disease[88], even if these patients cannot be distinguished preoperatively.

At the same time, in the eastern World, where D2 lymph node dissection is not a matter of debate, the challenge has been to demonstrate that super-extended lymph node dissection offers a better chance of a cure in gastric cancer treated with curative intent. The Taipei single-institution study that has compared D1 and D3 dissection has demonstrated a significant overall survival benefit in extended lymph node dissection, but no significant improvements in disease-free survival or in per-protocol analysis[90]. Moreover, the study showed that the morbidity of extended lymphadenectomy, although not lethal, is substantial even in experienced hands[95]. Finally, a multi-institutional, randomized and controlled trial by the Japan Clinical Oncology Group (JCOG-9501) has failed to demonstrate a survival benefit when super-extended (D2 + para-aortic node) lymph node dissection was performed. Moreover, in this randomized trial, the rate of postoperative morbidity in patients with a body mass index of $\geqslant$ 25 kg/m^2 and age > 65 years was a notable concern[96].

Geographical differences notwithstanding, all of these results agree with Cady's paradigm "...the therapeutic effect of cancer surgery is akin to that of a drug with a threshold or plateau effect: dose response up to a certain plateau, and then no further therapeutic effect beyond this point, only more complications"[97].

From a practical point of view, it is hard to believe that unresected overt nodal metastases in the tumor bed will not worsen prognosis. Likewise, it is hard to believe that resection of more negative lymph nodes will improve it. Tailoring lymph node dissection on the basis of actual lymph node involvement could be a key point for performing appropriate lymph node dissection and avoiding high rates of postoperative morbidity.

In the late 1980s, Kampschöer *et al*[98] developed software that was designed to match cases with characteristics similar to a given case. With seven demographic and clinical inputs, all identifiable preoperatively or intraoperatively, the program was able to predict the statistical likelihood of nodal disease for each of the 16 main nodal stations around the stomach[98-100]. The so-called "Maruyama Index of Unresected Disease" (MI), when retrospectively

used, was able to quantify the adequacy of lymphadenectomy. Such a novel measure was defined as the sum of Maruyama Program predictions for lymph node stations (Japanese stations 1-12) left *in situ* by the surgeon[101,102]. In a large United States adjuvant chemoradiation study, MI proved to be a strong predictor of survival that was independent from the level of lymph node dissection[103]. Furthermore, a blinded retrospective analysis of Dutch D1 *vs* D2 trial data has suggested that low-MI surgery is associated with significantly increased survival, regardless of lymph node dissection[104]. The MI aims to define an R+ lymph node dissection, and it appears that surgeons might have a better impact on single patient survival by pursuing a low MI operation (low probability of lymph node metastases left *in situ*) instead of relying exclusively on D-level guidance.

When the probability of lymph node metastasis is considered low, sentinel node dissection can be considered as another approach to customize lymph node dissection[105-107]. The sentinel nodes are the first sites of lymph node metastasis from a primary tumor and theoretically predict the involvement of more distant lymph nodes. To date, selective sentinel node dissection, detectable using the injection of either dyes or radioactive tracers, represents a standard procedure for melanoma and breast cancer with low probability of lymph node metastasis. In early gastric cancer, the risk of lymph node metastases is 2%-5% for patients with mucosal cancer and 11%-20% for those with submucosal cancer[108]. Sentinel node mapping results in gastric cancer have been variable since the lymphatic drainage from the stomach is very complicated and multidirectional, with an incidence of skip metastasis ranging from 5% to 10%[109]. Moreover, early reports have demonstrated that the loco-regional lymph node station contains truly positive nodes, even when the sentinel biopsy is negative. These anatomical peculiarities have led to the concept of a "sentinel lymphatic basin"[110], which indicates the lymph node stations to which sentinel nodes belong. Dissection of these stations can provide an acceptable safety net for the clinical application of these procedures, and minimize the possibility of leaving metastasis behind. Preliminary studies have shown that these sentinel node techniques are an acceptable procedure for pathological T1 tumors with a diameter of < 40 mm, although long-term follow-up data are still required[111-114].

R0 RESECTION: IS IT MERELY A SURGICAL TARGET?

Along with these classical surgical topics, in the past 20 years, three different modalities of adjuvant (pre- and postoperative) therapy have been proven to be effective by large-scale randomized trials. These include postoperative chemoradiation therapy (Unites States INT-0116 trial)[115], postoperative single-drug chemotherapy (Japanese ACTS-GC trial)[116] and perioperative three-drug combination chemotherapy (European MAGIC trial)[117]. Since the publication of these trials, surgery alone is no longer considered the standard treatment for patients with resectable locally advanced forms of gastric cancer, and the concept of radical resection needs to take into account the fact that R0 resection is not an exclusively surgical target.

Postoperative therapy: Recovery of R0 resection

Many studies and several meta-analyses with a focus on adjuvant postoperative chemotherapy have been conducted[118-127]. Summarizing their results, we can state that there is insufficient evidence, at present, to recommend postoperative chemotherapy as standard adjuvant treatment in Western patients. At present, these results should be cautiously managed, since these studies included very different patient populations, surgical procedures, and non-standardized timing and regimens of adjuvant therapy that are now considered as outdated[128]. At the same time, results from pivotal studies on postoperative chemoradiotherapy are inconclusive and conflicting because of the relatively small number of patients recruited[129-133].

In the United States, between 1991 and 1998, a study from the SWOG-Intergroup 0116 trial randomly assigned 556 patients to surgery only and surgery plus postoperative chemoradiotherapy: 45 Gy radiotherapy at 1.8 Gy/d, given 5 d/wk for 5 wk, with modified doses of fluorouracil and leucovorin on the first 4 d and last 3 d of radiotherapy. Two 5-d cycles of fluorouracil and leucovorin were given after, and one cycle was given before chemoradiotherapy[115]. Although clinically significant toxicity was recorded after chemoradiotherapy, the overall and relapse-free survival results of the surgery-alone arm were significantly worse than those of the adjuvant chemoradiotherapy arm. Chemoradiotherapy significantly improved median survival from 27 to 36 mo. Distant relapse was the most common pattern of recurrence in the adjuvant group (33% *vs* 18%), whereas local recurrence was more common in the surgery-only group (29% *vs* 19%). In this trial, < 10% of patients received formal D2 dissection, whereas 54% underwent D0 dissection. A common interpretation of these results is that adjuvant therapy may be useful in high-risk patients treated with inadequate lymph node dissection, because, through radiotherapy, it can eliminate residual lymph node metastasis, which would have been removed by D2 resection. A Korean non-randomized study[134] recently has shown that chemoradiotherapy after Japanese D2 resection improves survival. Currently, promising results from a randomized study conducted by the same group (SMC-IRB 2004-08-10 trial) are anticipated[135].

In 2007, the most convincing evidence on the benefits of adjuvant therapy was reported by the Japanese ACTS-GC trial (Adjuvant Chemotherapy Trial of TS-1 for Gastric Cancer)[116]. In this trial, 1059 patients with stage Ⅱ or Ⅲ gastric cancer who had undergone curative D2 gastrectomy were randomized to observation or 1-year administration of oral S-1. The study was terminated at the first interim analysis due to a highly significant difference in survival that favored chemotherapy. The incidence rate of loco-regional, lymphatic and peritoneal relapse was significantly lower in the chemotherapy arm than in the surgery-alone arm, although the rate of distant metastases

did not differ between the two arms. This study reported a survival-associated advantage with adjuvant chemotherapy within the context of surgery performed according to Japanese standards.

New ongoing trials investigating adjuvant therapy (CLASSIC trial, SMC-IRB 2004-08-10, CALGB-80101) are expected to show the true efficacy and survival benefits in the near future[135-137].

In the past 30 years, Japanese and Korean researchers have performed a number of trials that have investigated the use of immunochemotherapy as adjuvant treatment after curative resection of gastric cancer. A variety of immunotherapeutic agents, such as protein-bound polysaccharide (polysaccharide K extracted from mycelia of *Coriolus versicolor*; PSK)[138,139], *Streptococcus pyogenes* preparation (OK-432)[140,141], polysaccharide sizofiran[142], *Nocardia rubra* cell wall skeleton[143], Bacillus Calmette-Guerin (BCG)[144] and polyadenylic-polyuridylic acid[145] have been used in addition to chemotherapy.

Results from randomized trials that have compared adjuvant chemo-immunotherapy with surgery alone or with other chemotherapeutic schedules have been contradictory because of a lack of robust evidence in clinical practice[146]. However, interesting results have been derived from two recent meta-analyses about OK-432 and PSK[147,148].

The benefit of combined adjuvant chemotherapy and immunotherapy with OK-432 (a lyophilized preparation of a low virulence group A *S. pyogenes*), in patients with curatively resected gastric cancer was assessed by Sakamoto *et al*[147] in a meta-analysis of data derived from 1522 patients enrolled in six randomized clinical trials. In these trials, adjuvant chemotherapy, usually consisting of induction with mitomycin C plus long-term oral fluordinated pyrimidines, was compared with the same chemotherapy plus OK-432. The 3-year survival rate for all eligible patients in the six trials was 67.5% in the chemo-immunotherapy group *vs* 62.6% in the chemotherapy-only group. The 3-year overall survival odds ratio was 0.81 (95% CI: 0.65-0.99). The beneficial treatment effect was shown to be statistically significant ($P < 0.044$). The results of this meta-analysis were interpreted by the authors to suggest that chemo-immunotherapy after surgery with OK-432 can improve the survival of patients with successfully resected gastric cancer.

The effect of adjuvant immunochemotherapy with PSK after curative resection of gastric cancer by means of a meta-analysis of eight randomized trials has been assessed by Oba *et al*[148]. In this analysis, the estimated overall HR was 0.88 (95% CI: 0.79-0.98, $P = 0.018$) with no significant heterogeneity between the treatment effects observed in different studies. The authors have concluded that the addition of PSK to standard chemotherapy offers significant advantages in survival over chemotherapy alone for patients with curative resections of gastric cancer.

Also for postoperative chemo-immunotherapy, there is a necessity for clear evidence in future studies; particularly, the clinical use of immunostimulating factors should be tested in large randomized trials.

Pre-/perioperative therapy: Induction of R0 resection

The rationale for preoperative therapy is based on several theoretical assumptions. Preoperative antiblastic therapy might reduce the risk of proliferation and allow for *in vivo* chemosensitivity tests, thus facilitating the choice of the most appropriate postoperative regimen. Furthermore, the preoperative approach has two distinct advantages: increased compliance due to an undoubtedly better performance status in patients who are not burdened with surgical complications, nutritional impairment, or damaged vascularization of the tumor bed. The twofold goal of eliminating hidden micrometastases along with tumor down-staging might increase the probability of a truly curative complete resection with delayed surgery.

Investigation of the efficacy and possible uses of chemotherapy in patients with advanced gastric cancer began in the late 1970s[149-151]. Encouraging results, however, were not reported until the early 1990s, when two independent studies in patients with non-resectable disease found that chemotherapy led to subsequent resection in 40%-50% of patients, with an increase in total median survival of 18 mo, compared with unresected patients[152,153]. These preliminary observations encouraged the introduction of preoperative chemotherapy protocols for potentially resectable, locally advanced gastric cancer (Table 1)[117,154-166]. However, the results of these first trials are questionable, mainly because of their methodological limitations. By following an inaccurate preoperative staging process, several authors have recruited patients on non-homogeneous criteria, commonly recruiting patients with locally advanced gastric cancer and others with disease of unclear stages, without a fixed distinction between resectable and non-resectable tumors. In addition to non-homogeneous methods of recruitment, other sources of bias in early trials included the use of different chemotherapeutic regimens, non-standardized surgery or surgery of questionable quality, and missing or poorly detailed response criteria.

In 1993, the Dutch Gastric Cancer Group started the first randomized controlled trial of exclusively preoperative chemotherapy for gastric cancer (cardia tumors were excluded)[161]. The regimen used was FAMTX (fluorouracil, doxorubicin, and methotrexate), which was, at that time, the gold standard of treatment for adenocarcinoma of the stomach. This trial had many accrual problems and was prematurely stopped after an interim analysis showed that FAMTX was unlikely to achieve the goal of a 15% increase in curative resectability after preoperative chemotherapy. Several biases have been outlined for this study, particularly the inaccuracy of the staging procedure with optional use of CT and laparoscopy, and inadequate extension of lymphadenectomy. The investigators reported a high rate of tumor progression during treatment (36%) along with a reduction in curative resections (56% *vs* 62%) and a decreased median survival (18 mo *vs* 30 mo), compared with untreated patients. Even if all of the statistical differences in this study were insignificant, both the short-term and long-term results were discouraging[161,167].

Since the late 1990s, ambitious European phase Ⅲ

Table 1 Trials of preoperative chemotherapy in gastric cancer

Author	Phase	Selection criteria	Preoperative	Postoperative	Pts	R0 (%)[1]	Pathological CR (%)	Median survival (mo)
Ajani *et al*[154], 1991	Ⅱ	M0 *Resectable* (+ GEJ)	EFP × 2	EFP × 3	25	72	0	15
Leichman *et al*[155], 1992	Ⅱ	M0 *Resectable*	FPL × 2	IP FUDR + P cisplatin × 2	38	88	8	> 17
Kang *et al*[156], 1992	Ⅲ RCT	M0 *Loc. advanced*	EFP × 3	EFP × 3-6	53	79	8	43
			None		54	61	-	30
Ajani *et al*[157], 1993	Ⅱ	M0 *Resectable*	EAP × 3	EAP × 2	48	90	0	16
Rougier *et al*[158], 1994	Ⅱ	M0 *Loc. advanced* (+ GEJ)	FP × 6	None	30	78	0	16
Kelsen *et al*[159], 1996	Ⅱ	M0 *Loc. advanced*	FAMTX × 3	IP FP + F	56	77	NS	15
Crookes *et al*[160], 1997	Ⅱ	M0 *Resectable* (+ GEJ)	FPL × 2	IP FUDR + IP cisplatin × 2	59	71	9	52
Songun *et al*[161], 1999	Ⅱ RCT	T2-T4; M0	FAMTX × 4	None	27	75	NS	18
			None		29	75	-	30
Schuhmacher *et al*[162], 2001	Ⅱ	Ⅲ-Ⅳ; M0 (+ GEJ)	EAP	None	42	86	0	19
D'Ugo *et al*[163], 2006	Ⅱ	T3-T4 anyN; T ≤ 2 N+; M0	EEP × 3 or ECF × 3	EEP × 3 or ECF × 3	34	82	3	> 28
Cunningham *et al*[117], 2006	Ⅲ RCT	Ⅱ-Ⅳ; M0 (+ GEJ)	ECF × 3	ECF × 3	250	74	NS	18
			None	None	253	68	-	30
Boige *et al*[164], 2007	Ⅲ RCT	*Resectable* (+ GEJ)	FP × 3	FP × 3	113	84	NS	NS
			None	None	111	73	-	
Schuhmacher *et al*[165], 2009	Ⅲ RCT	*Loc. advanced* T3-T4NxM0	FP × 2	None	72	81.9	NS	> 36
			None		72	66.7		
Kinoshita *et al*[166], 2009	Ⅱ	Schirrous *Resectable*	TS-1 × 2	None	55	80.8	0	NS

[1]The "R0" resection rate was calculated only among resection procedures. Pts: Number of patients recruited R0, curative (R0) resections; CR: Complete response; GEJ: Gastroesophageal junction; EFP: Etoposide, fluorouracil, and cisplatin; FPL: Fluorouracil, cisplatin, and leucovorin; IP: Intraperitoneal; FUDR: 5-fluoro-2'-deoxyuridine; RCT: Randomized controlled trial; EAP: Etoposide, doxorubicin, and cisplatin; FP: Fluorouracil and cisplatin; FAMTX: Fluorouracil, doxorubicin, and methotrexate; F: Fluorouracil; NS: Not stated; EEP: Etoposide, epirubicin and cisplatin; ECF: Epirubicin, cisplatin, and fluorouracil.

trials have been designed to provide a definitive demonstration of the efficacy of preoperative treatments. The adoption of strict selection criteria made the selection of patients so difficult that some studies were stopped prematurely (EORTC 40954 and SWS-SAKK-43/99 trials)[165,168]. Only the MAGIC trial (started in the United Kingdom in 1994) and the FFCD 9703 trial (started in France in 1996) have been completed[117,164]. These two studies have yielded substantial evidence supporting the efficacy of perioperative chemotherapy for an increased survival rate (36% *vs* 23%, estimated at 5 years for MAGIC, 38% *vs* 24% estimated at 5 years for FFCD 9703, Table 1), along with a significantly higher curative resection rate in the treated group *vs* the surgery-alone group (79% *vs* 70%, $P = 0.03$ for MAGIC, 84% *vs* 73% in arm 2, $P = 0.04$ for FFCD 9703) without an increase in perioperative morbidity or mortality.

The possible increase in the actual R0-resection rate has been an important goal of preoperative chemotherapy. In a phase Ⅱ study of a perioperative chemotherapy protocol, the achievement of R0 resection in response to preoperative chemotherapy was shown to be the most significant prognostic indicator by univariate and multivariate analysis. Furthermore, R0 resection was the only independent variable in determining the probability of long-term survival in locally advanced gastric carcinoma. The overall survival for all curatively resected patients is higher when compared to historical series treated with surgery alone for locally advanced gastric cancer[163,169].

Based on the results of the SWOG 9008/INT-0116 trial[115], the integration of chemotherapy with radiation applied in the preoperative phase has gained much interest. Some benefits of preoperative radiotherapy for gastric cancer have been reported by a pivotal randomized single-center Chinese study by Zhang *et al*[170]. This study recruited 317 patients with adenocarcinoma of the cardia that were randomly assigned to radiotherapy followed by surgery, or surgery alone. This study indicated a significant 5-year survival benefit for patients treated with neoadjuvant radiotherapy as compared with surgery alone (30.1% *vs* 19.8%, respectively), with an improved rate of complete curative resection after radiotherapy (80% *vs* 62%). Recently, published phase Ⅱ studies have verified the efficacy of chemoradiotherapy in terms of complete pathological response (up to 30% in some series) and increased long-term survival without an increase in morbidity or mortality[171-174].

All of the above results suggest that R0 resection is not an exclusive surgical target in locally advanced gastric cancer, but that it can be facilitated or achieved by preoperative therapy (induction of R0 resection).

Many answers are expected from ongoing trials exploring ways of improving preoperative treatment strategies for resectable gastric cancer: the MAGIC B trial (United Kingdom National Cancer Research Institute ST03 trial) of perioperative epirubicin, cisplatin, and capecitabine, with or without the endothelial growth factor antibody, bevacizumab[175]; the CRITICS trial (Chemo-Radiotherapy after Induction chemoTherapy In Cancer of the Stomach), a phase Ⅲ study that is randomizing between preoperative chemotherapy (three courses of epirubicin/cisplatin/capecitabine) and gastric surgery with

limited lymph node dissection followed by postoperative chemotherapy (another three courses of epirubicin/cisplatin/capecitabine) or chemoradiotherapy[176]; and the JCOG trial 0501 (Japan Clinical Oncology Group Study 0501 trial) and KYUH-UHA-GC04-03 Kyoto trial, which are testing preoperative oral fluoropyrimidine S-1 together with cisplatin *vs* postoperative oral fluoropyrimidine S-1[177].

CONCLUSION

In gastric cancer, radical resection (R0 resection) offers the best chance for a cure because it is defined as the complete surgical removal of any residual cancer cells in the tumor bed. However, distant and loco-regional failure rates in most radically resected patients with positive lymph nodes or involvement of the serosa contradict this statement.

All current therapeutic efforts in resectable gastric cancer are directed toward the individualization of therapeutic protocols, which tailors the extent of resection and the administration of pre- and postoperative treatment. A paradigm shift has rapidly advanced in the past 10 years: three pivotal studies in three different areas of the world (United States, Europe and Japan) have demonstrated that multimodal treatments improve the prognosis for patients with resectable gastric cancer. The common target of all of these strategies is to improve prognosis towards the achievement of a true curative resection (R0 resection) with minimal morbidity and mortality.

In gastric cancer, surgical research has always proceeded slowly, and standardization is still far from being settled. Geographical differences in epidemiology and treatment approaches and a lack of surgical gold standards have diverted attention from the pursuit of a multimodal approach. In other solid neoplasms worldwide, the multimodal approach has already been validated. In the near future, we expect the same to occur for gastric cancer, provided that the published evidence that is needed to reach this goal is further improved and developed. The result of treatment for locally advanced gastric cancer is the sum of the effect of local tumor control by surgery, with or without radiotherapy and/or systemic chemotherapy. The role of each treatment modality varies according to the stage of the disease, individual patient risk, surgical volume, available chemotherapy regimens and quality of radiotherapy. Evidence of the effect of different combinations of treatments should be established for each clinical circumstance, and surgeons should play a key role in this.

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ORIGINAL ARTICLE

Effect of soy saponin on the growth of human colon cancer cells

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Abstract

AIM: To investigate the effect of extracted soybean saponins on the growth of human colon cancer cells.

METHODS: WiDr human colon cancer cells were treated with 150, 300, 600 or 1200 ppm of soy saponin to determine the effect on cell growth, cell morphology, alkaline phosphatase (AP) and protein kinase C (PKC) activities, and P53 protein, c-Fos and c-Jun gene expression.

RESULTS: Soy saponin decreased the number of viable cells in a dose-dependent manner and suppressed 12-O-tetradecanol-phorbol-13-acetate-stimulated PKC activity ($P < 0.05$). Cells treated with saponins developed cytoplasmic vesicles and the cell membrane became rougher and more irregular in a dose-dependent manner, and eventually disassembled. At 600 and 1200 ppm, the activity of AP was increased ($P < 0.05$). However, the apoptosis markers such as c-Jun and c-Fos were not significantly affected by saponin.

CONCLUSION: Soy saponin may be effective in preventing colon cancer by affecting cell morphology, cell proliferation enzymes, and cell growth.

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Key words: Soy saponin; Colon cancer; Apoptosis; Cell proliferation; Cell differentiation

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INTRODUCTION

Colon cancer is one of the major causes of cancer mortality worldwide, which results from interactions of different factors such as aging, family history and dietary style. It has been suggested that consumption of higher levels of soy foods can lead to a lower incidence of acquiring colon cancer[1,2]. The most discussed compounds related to colon cancer in soy are isoflavone and saponins[3]. Saponins have been found to have biological benefits and have been utilized pharmaceutically[4]. Soy saponins are amphiphilic compounds and categorized as triterpenoic saponins. They are able to interact with the cancer cell membranes that are rich in phospholipids and cholesterol and with the hydroxyl groups on the aglycone moiety[5]. Research has found that steroid saponins extracted from fenugreek reduce the number of aberrant crypt foci in azoxytethane-induced rat colon cancer, and induce apoptosis of HT-29 human colon cancer cells[6]. However, whether soy saponins affect the growth of cancer cells by causing apoptosis or necrosis is still not clear.

In this study, we investigated the *in vitro* physical and biological effects of soy saponins on WiDr colon cancer cells, the same cell line as HT-29[7] (American Type Culture Collection, Rockville, MD, USA; Catalogue 1988), by

examining the number of living cells, cell morphology, alkaline phosphatase (AP) and protein kinase C (PKC) activities, and the expression of c-Jun, c-Fos, and P53 protein, and cell apoptosis.

MATERIALS AND METHODS

Soy saponin preparation and analysis

Saponin extraction was performed according to the method of Berhow *et al*[8]. The purity of crude saponin extracted was examined by high performance liquid chromatography (HPLC) (TSP, Germany) using commercial soy saponin as a standard (Wako, Japan). The HPLC conditions were as follows: C18 column (Vercopak, ODS-3, 4.6 mm × 250 mm); UV absorbance: 190-350 nm; analyzing temperature 30℃; flow rate: 1 mL/min; gradient solvent system: solvent A, 0.05% trifluoroacetic acid in water, solvent B, acetonitrile; 63% A to 52% A in 38 min.

Cell culture and viability

Cells were cultivated in minimal essential medium that contained 10% fetal bovine serum, sodium bicarbonate (1.5 g/L), and 1.0 mmol/L sodium pyruvate at 37℃ and 5% CO_2. Cells were subcultured into a new medium (100 mm diameter dish) when they reached a high density, at a series of dilutions from 1:3 to 1:6. When they reached 2×10^3 cells/well, cells were cultivated in each well of a 96-well plate. After stable attachment in the medium (day 0), cells were treated with five different concentrations (0, 150, 300, 600, 1200, 2400 ppm) of soy saponins (16 wells/concentration). CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, USA) was used to measure the viability of cells every 24 h for 3 d.

Cell morphology observation

After the cells were treated with different concentrations of soy saponins for 24 h, they were examined by electron microscopy. Scanning electron microscopy (SEM; S-2400; Hitachi, Japan) was performed to observe the differences in cell morphology. Transmission electron microscopy (TEM; H600; Hitachi) was used to investigate intracellular morphology.

Cell proliferation/differentiation measurement

Cells were cultivated in a 10-cm Petri dish. After the cells were stable, fresh medium with 0, 150, 300, 600, and 1200 ppm of soy saponins was added. Cells were cultivated for another 72 h before being subjected to tests for proliferation and differentiation. Sodium butyrate (2.5 mmol/L) was used as a positive control for detecting AP activity. The level of differentiation was measured using Alkaline Phophatase Liquicolor (Human, Germany). The activity of PKC was measured using Peptag® Assay (Promega, USA). c-Jun, c-Fos, and wild-type P53 protein expression in WiDr cells was analyzed using SDS-PAGE and western blotting. A 12% resolving gel and a 5% stacking gel were applied. β-actin (43 kDa) was used as the internal control. The antibodies used were rabbit anti-c-Jun polyclonal antibody (Stressgen, Canada), rabbit anti-c-Fos polyclonal antibody, mouse anti-P53 monoclonal antibody, and mouse anti-β-actin monoclonal antibody (Sigma, USA).

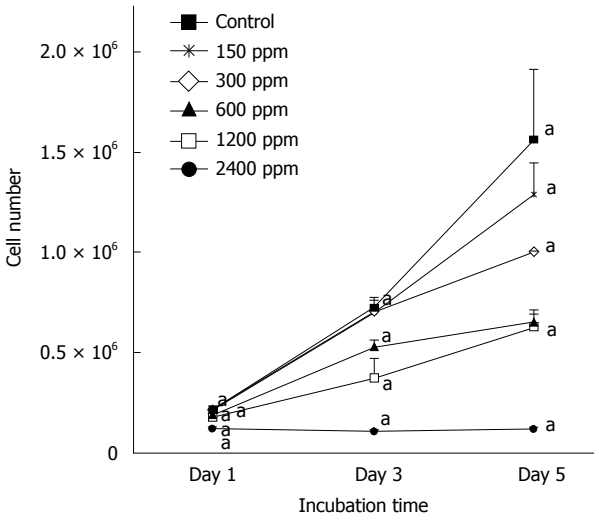

Figure 1 Effect of extracted crude soybean saponins on the growth of WiDr cells. Different concentrations of saponin at each incubation time were compared using one way analysis of variances with Fisher's test. Values are mean ± SD. Points with letter "a" represent significant differences at the $P < 0.05$ level.

Statistical analysis

Data were expressed as mean ± SD. One-way analysis of variances and Fisher's least significant difference were performed using SAS 8.13. Differences were significant at $P < 0.05$.

RESULTS

Soy saponins and cell count

Figure 1 illustrates the dose-dependent inhibitory effect of soy saponins on the number of WiDr cells. At the end of day 1, the cell count was significantly lower in the group treated with 2400 ppm saponins compared to that in the control group ($P < 0.05$). The percentage inhibition was 40.7%. At the end of day 3, compared to the control group, the number of cells in the groups treated with 600, 1200 and 2400 ppm was significantly lower ($P < 0.05$), with percentage inhibition of 27.4%, 56.6% and 84.8%, respectively. At the end of day 5, the groups treated with 300, 600, 1200 and 2400 ppm of soy saponins had a lower cell count than the control group ($P < 0.05$), with percentage inhibition of 36.0%, 57.9%, 59.7% and 92.2%, respectively. Under light microscopic observation at the end of day 5, it was shown that cell density in the medium decreased in parallel with the increase of soy saponins in the medium (Figure 2). Figure 3 shows that under treatment with soy saponins (150-2400 ppm), the percentage cell survival was decreased in a reversed dose-dependent manner ($P < 0.05$) at each time point.

SEM of WiDr cells

Figure 4A-D shows the SEM observation of WiDr cells treated with 0, 300, 600, 1200 and 2400 ppm of soy saponins. When the dose of soy saponins increased, the

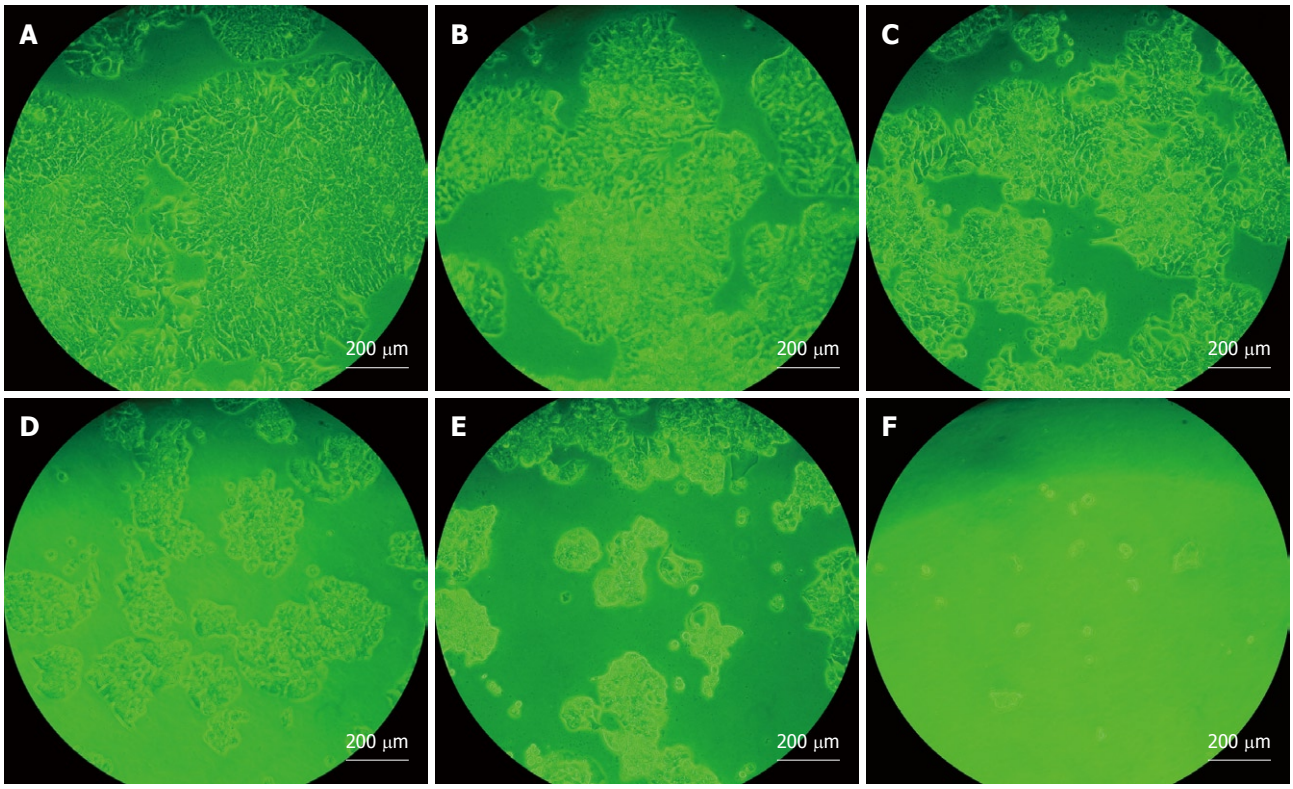

Figure 2 Cell number and morphological effects of extracted crude soybean saponins on WiDr cells. A: Untreated control culture for 5 d; B: Culture exposed to 150 ppm extracted crude soybean saponins for 5 d; C: 300 ppm; D: 600 ppm; E: 1200 ppm; F: 2400 ppm.

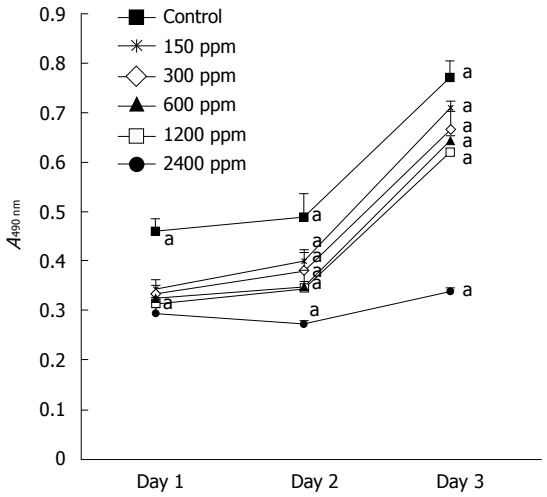

Figure 3 Effect of extracted crude soybean saponins on viability of WiDr cells. Different concentrations of saponin at each incubation time were compared using one way analysis of variances with Fisher's test. Values are mean ± SD. Points with letter "a" represent significant differences at the $P < 0.05$ level.

surface of WiDr cells became rougher, and the cell shape changed from round to irregular. As the dose reached 1200 ppm (Figure 4C), breaks were seen on the surface of WiDr cells. At 2400 ppm soy saponin, complete deformation of WiDr cells was observed (Figure 4D).

Activity of AP

Figure 5 shows that soy saponins induced AP activity in WiDr cells in a dose-dependent manner ($P < 0.05$). The WiDr cell line is one of the colon cancer cell lines without AP activity. The control sample with sodium butyrate showed increased AP activity, while the one without sodium butyrate did not. The activated AP indicated that WiDr cancer cells might slow down the proliferation process but shift toward the differentiation process.

Activity of PKC

The effect of soy saponin on PKC activity is shown in Figure 6. 12-O-tetradecanoyl phorbol-13-acetate (TPA) was added to the medium to induce PKC activity. The medium without TPA showed no PKC activity. As the dose of saponins in the medium increased, the inhibitory effect on PKC increased.

Expression of P53, c-Jun, and c-Fos

There was no significant difference in the expression of P53 and c-Fos proteins between the groups with/without soy saponin treatment (data not shown). On the other hand, Figure 7 shows a trend towards an inhibitory effect of saponins on the expression of c-Jun after 3 d of incubation.

DISCUSSION

In this study, we investigated the effects of soy saponin on cell growth, proliferation/differentiation-related enzyme activities, and the expression of apoptosis-related proteins of WiDr human colon cancer cells. We found that soy saponins effectively inhibited the growth rate and survival

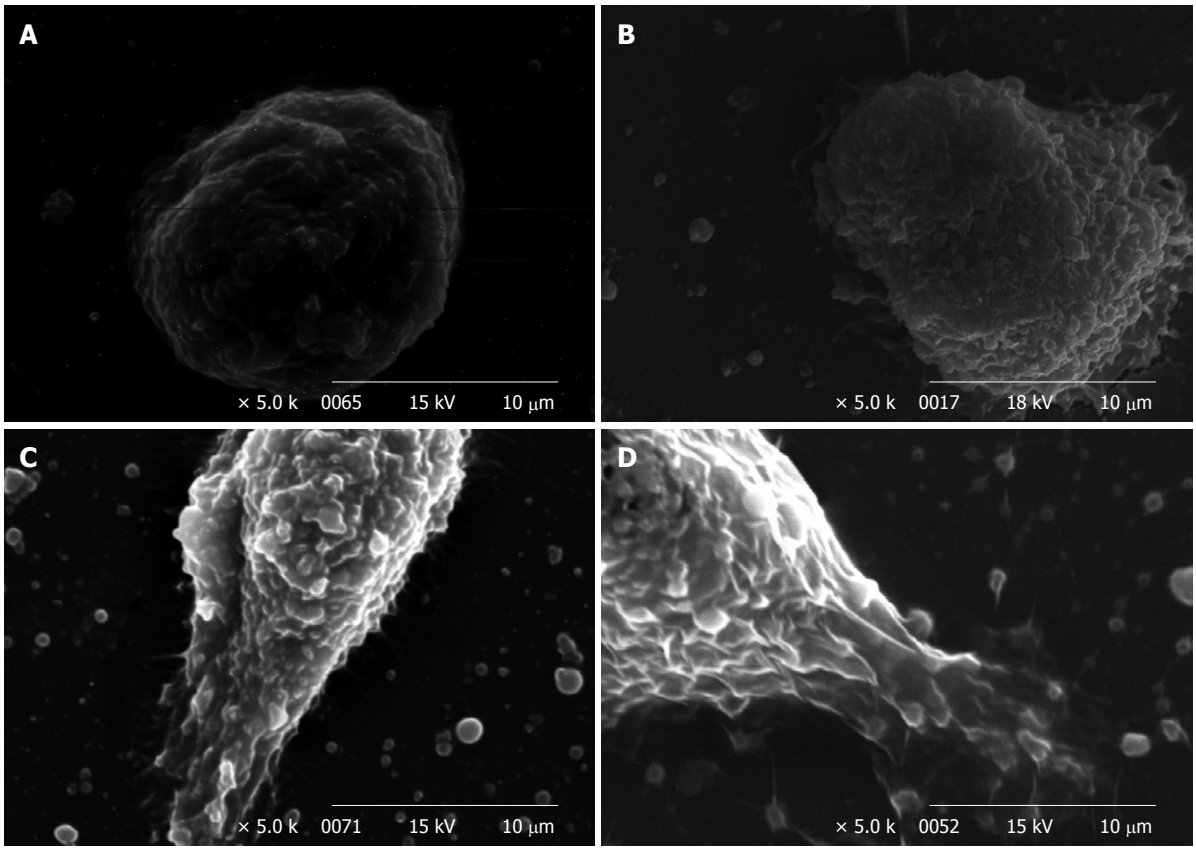

Figure 4 Scanning electron microscopy electron micrographs of WiDr cells. Cells were treated with 0 (A), 300 (B), 1200 (C) and 2400 ppm (D) soy saponin, for 1 d.

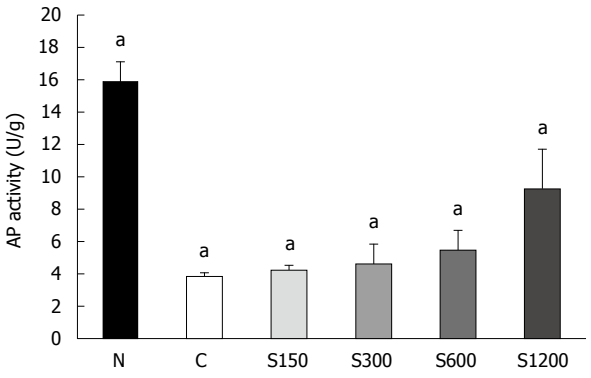

Figure 5 Effect of extracted crude soybean saponins on alkaline phosphatase activity of WiDr cells. N: Culture exposed to 2.5 mmol/L sodium butyrate for 3 d; C: Untreated control culture for 3 d; S150: Culture exposed to 150 ppm extracted crude soybean saponins for 3 d; S300: 300 ppm; S600: 600 ppm; S1200: 1200 ppm. Different concentrations of saponin at each incubation time were compared using one way analysis of variances and Fisher's least significant difference test. Values are mean ± SD. Bars with letter "a" represent significant differences at the $P < 0.05$ level.

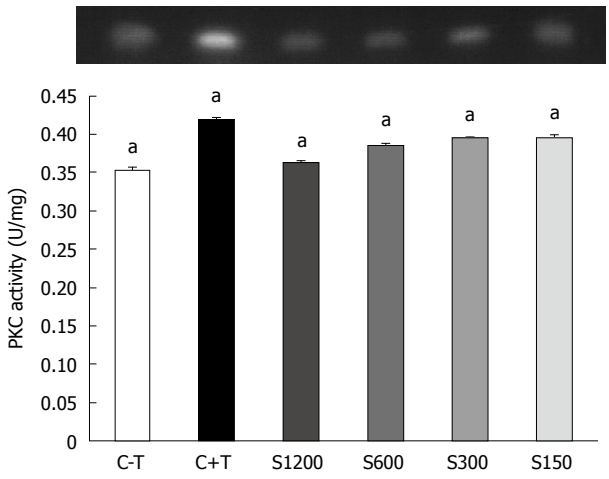

Figure 6 Effect of extracted crude soybean saponins on protein kinase C activity of WiDr cells. C-T: Untreated control culture for 3 d; C+T: Control culture + 100 ng/mL tetradecanoyl phorbol-13-acetate (TPA); S150: Culture exposed to 150 ppm extracted crude soybean saponins + 100 ng/mL TPA for 3 d; S300: 300 ppm; S600: 600 ppm; S1200: 1200 ppm. Values are mean ± SD. Different concentrations of saponin at each incubation time were compared using one way analysis of variances and Fisher's least significant difference test. Bars with letter "a" represent significant differences at the $P < 0.05$ level.

of human colon cancer cells in a dose-dependent manner. Soy saponins are amphiphilic compounds that can be used as bio-surfactants. They are structurally similar to oleanolic acid and ursolic, which have been shown to be glucocorticoid receptors with anti-carcinogenic activity[9,10].

PKC is one of the markers for cell proliferation. PKC activity increases as the cells undergo the proliferation process. As shown in our study, the addition of soy saponin effectively inhibited the activity of PKC in a dose-dependent manner. On the other hand, P53 protein is responsible for regulating some reactions such as the cell cycle, DNA repair, and apoptosis[11,12]. The relationship between P53 protein and the HT-29 cell death is still not clear[13]. Shen *et al*[14] have found that 2'-OH flavanone inhibits the growth of HT-29 cells *via* increasing the expres-

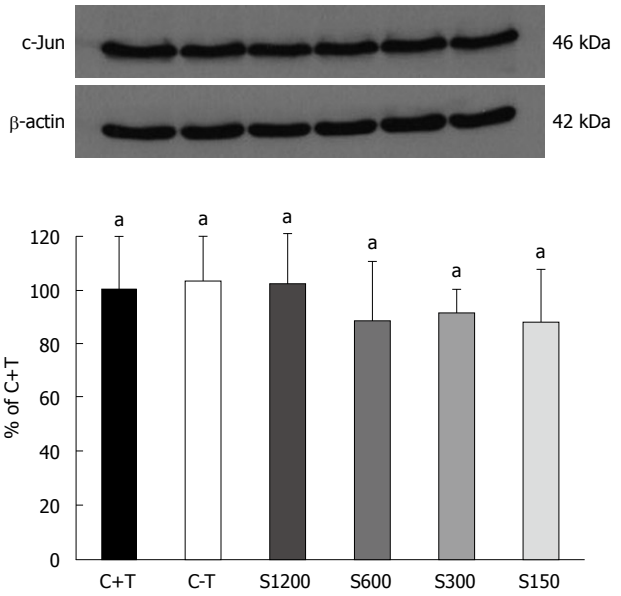

Figure 7 Effect of crude soybean saponins extracted on c-Jun (46 kDa) expression of WiDr cells. C+T: Control culture + 100 ng/mL tetradecanoyl phorbol-13-acetate (TPA); C-T: Untreated control culture for 3 d; S150: Culture exposed to 150 ppm extracted crude soybean saponins + 100 ng/mL TPA for 3 d; S300: 300 ppm; S600: 600 ppm; S1200: 1200 ppm. Values are mean ± SD. Different concentrations of saponin at each incubation time were compared using one way analysis of variances with Fisher's test. Bars with letter "a" represent significant differences at the $P < 0.05$ level.

sion of P21, but it has no effect on P53 protein. In our study, we did not find any inhibitory effect of soy saponin on the P53 protein of WiDr cells.

Under normal conditions, cells proliferate to a certain level and then differentiate to different kinds of cells. If the cells are stimulated by some exogenous factors, for example, carcinogens, cells may not differentiate, proliferate abnormally, and form tumors. In our study, compared to the control group, the cell number was decreased and AP activity was increased by addition of soy saponins, which is an indication of cell differentiation. It has been shown that materials such as vitamin D3, with membranolic actions, can regulate the transportation of Ca^{2+} ions through the membrane and induce cell differentiation[15]. Our SEM results showed that cell morphology was changed by saponins, with a similar membranolic effect. Soy saponins may also promote cell differentiation if the cell membrane has not been destroyed by too high a concentration.

Programmed cell death can be categorized into two types, type I apoptosis and type Ⅱ autophagic death[16-18]. The major differences in these two types are that, in apoptosis, cells die individually, and phagocytes are necessary for cell degradation. On the other hand, in autophagic death, cells die in groups through a lysosomal mechanism, in which vacuoles are observable in cells[17]. In our SEM study, cell membranes became rough and wrinkled when treated with high concentrations of saponins. In addition, under TEM observation (Figure 8), vacuoles appeared in the cells that had been treated with higher concentrations of saponins, which may be an indication of type Ⅱ autophagic death. It has been found that the level of microtubule-associated protein light chain 3 is increased after

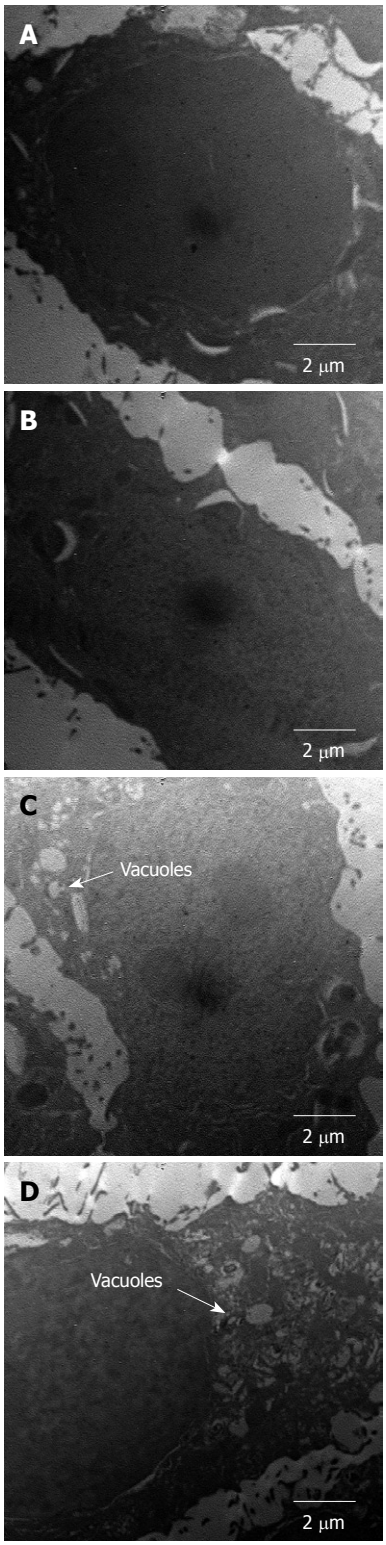

Figure 8 Transmission electron microscopy electron micrograph of WiDr cells treated with 150 (A), 300 (B), 600 (C), and 1200 (D) ppm of extracted crude soybean saponins for 1 d. At 600 and 1200 ppm of saponin, vacuoles were observable.

saponin treatment[19], which is an indication of type Ⅱ autophagic death. For these reasons, the inhibitory effect of soy saponins on WiDr cells may not be apoptosis, but rather autophagic death at higher concentrations.

In conclusion, we found that soy saponins changed the membrane structure and affected the growth of WiDr

cells in two different ways; by increasing the AP activity while reducing PKC activity to induce cell differentiation at lower concentrations, or by inducing type Ⅱ autophagic death at higher concentrations. This may need further investigation.

COMMENTS

Background

Colon cancer is one of the major causes of cancer mortality worldwide. Soy saponins are categorized as amphiphilic compounds, and may be able to react with the phospholipids and cholesterol on the membrane of cancer cells, and with the hydroxyl groups on the aglycone moiety.

Research frontiers

Steroid saponins extracted from fenugreek reduced the number of colon aberrant crypt foci in azoxymethane-induced rat colon cancer and induced apoptosis of HT-29 human colon cancer cells. However, how soy saponins affect the growth of cancer cells is still not clear. In this study, the authors investigated the *in vitro* physical and biological effects of soy saponins on WiDr colon cancer cells.

Innovations and breakthroughs

Recent studied have suggested that saponins affect the growth of colon cancer cells. This is believed to be the first thorough study that has focused on the relationship between biomarkers of apoptosis, such as expression of c-Jun, c-Fos, and P53 protein, and cell morphology, proliferation, and differentiation.

Applications

By understanding how soy saponins affect colon cells, this study may represent a future strategy for prevention or treatment of colon cancer.

Peer review

The authors investigated the inhibitory effects of soy saponins on colon cancer cells. Soy saponins inhibited the growth of colon cancer cells by reducing protein kinase C activity, while the features of type Ⅱ programmed cell death (autophagic death) was observed. It is a well written paper with promising results that may be the basis of forthcoming research in cancer biology and therapy.

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ORIGINAL ARTICLE

Galangin induces apoptosis of hepatocellular carcinoma cells *via* the mitochondrial pathway

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Abstract

AIM: To investigate the mechanism by which galangin, a polyphenolic compound derived from medicinal herbs, induces apoptosis of hepatocellular carcinoma (HCC) cells.

METHODS: The 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay was used to measure cell viability. Apoptosis was evaluated by *in situ* uptake of propidium iodide and Hoechst 33258 and was then detected by fluorescence microscopy. Protein expressions were detected by Western blotting. To confirm the apoptotic pathway mediated by galangin, cells were transfected by *bcl-2* gene to overexpress Bcl-2 or siRNA to down-regulate Bcl-2 expression.

RESULTS: Galangin (46.25-370.0 μmol/L) exerted an anti-proliferative effect, induced apoptosis, and decreased mitochondrial membrane potential in a dose and time-dependent manner. Treatment with galangin induced apoptosis by translocating the pro-apoptotic protein Bax to the mitochondria, which released apoptosis-inducing factor and cytochrome c into the cytosol. Overexpression of Bcl-2 attenuated galangin-induced HepG2 cell apoptosis, while decreasing Bcl-2 expression enhanced galangin-induced cell apoptosis.

CONCLUSION: Our data suggests that galangin mediates apoptosis through a mitochondrial pathway, and may be a potential chemotherapeutic drug for the treatment of HCC.

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Key words: Hepatocellular carcinoma; Galangin; Mitochondria; Bcl-2; Apoptosis

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Zhang HT, Luo H, Wu J, Lan LB, Fan DH, Zhu KD, Chen XY, Wen M, Liu HM. Galangin induces apoptosis of hepatocellular carcinoma cells *via* the mitochondrial pathway. *World J Gastroenterol* 2010; 16(27): 3377-3384 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3377.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3377

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, particularly in China. Surgical therapy, chemotherapy, and radiation have been used for the treatment of HCC. However, HCC remains one of the

more difficult cancers to treat. Although chemotherapy is a common therapeutic strategy after surgery, its use has been toxic to normal tissues and limits their use. Therefore, it is important to screen for new anti-cancer drugs.

Recent studies show that a number of dietary compounds possess anti-cancer properties[1], including curcumin, genistein, quercitin, resveratrol, piceatannol and pterostilbene. These dietary compounds may enhance the efficacy of chemotherapeutic agents by modifying the activity of specific targets that control cell proliferation and apoptosis such as Bcl-2, Bcl-xL, X-linked inhibitor of apoptosis protein[2], Akt[3], c-myc, neuronal apoptosis inhibitory protein, c-IAP-2, nuclear factor-κB[4], survivin, p21WAF1 and p53[5].

Galangin (4H-1-Benzopyran-4-one, 3,5,7-trihydroxy-2-phenyl-), a flavonoid, is a polyphenolic compound derived primarily from medicinal herbs, including Alpinia officinarum Hance, Alnus pendula Matsum, Plantago major L, and Scutellaria galericulata L. (S. scrodifolia Fisch.). Eaton *et al*[6] demonstrated that galangin could inhibit the methoxyresorufin *O*-demethylase activity of CYP1A2, CYP1A1 and P-form phenolsulfotransferase. These observations suggest galangin may be a potential chemopreventive agent in sulfation-induced carcinogenesis.

Furthermore, Moon has reported that galangin had cancer preventive properties and induced apoptosis in several cancer cell lines[7]. Another study showed that galangin could induce a G0-G1 cell cycle arrest, modulate the expression of cycline/cdk, and decrease Bcl-2 levels, which leads to apoptosis of imatinib-resistant Bcr-Abl expressing chronic myelogenous leukemia cell lines[8].

Based on these previous reports, it was suggested that galangin could inhibit cell proliferation and induce apoptosis. Therefore, galangin may be a potential anti-tumor agent. However, the mechanism by which galangin exert its anti-tumor activity is unknown. In this study, we demonstrate the effects of galangin on HCC cells and elucidate the mechanism by which galangin induces apoptosis.

MATERIALS AND METHODS

Cell culture

Three human liver cancer cell lines (HepG2, Hep3B and PLC/PRF/5) were obtained from American type culture collection (Rockville, MD, USA) and kept in Institute of Biochemistry and Molecular Biology, Guangdong Medical College. All cell lines were cultured in Dulbecco's modified Eagle medium (Gibco BRL, Grand Island, NY, USA) containing penicillin (100 μg/mL) and streptomycin (100 μg/mL) and supplemented with 10% fetal bovine serum (Sijiqing Laboratories, Hangzhou, China) at 37℃ in a humidified atmosphere with 5% CO_2.

Agents and chemicals

Galangin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO), with the final concentration of DMSO in the culture medium below 0.1% (v/v). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Hoechst 33258, propidium iodide, rhodamine 123 [2-(6-amino-3-imino-3*H*-xanthen-9-yl) benzoic acid methyl ester] were purchased from Sigma (St. Louis, MO, USA). Rabbit or goat polyclonal antibodies against Bax, caspase-3, caspase-9, cytochrome c, apoptosis-inducing factor (AIF), PAPR, Vox Ⅳ, GAPDH and Bcl-2 were from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA, USA).

MTT viability assay

HCC cells (5.0×10^3) were seeded and treated with different concentrations of galangin or different times in 96-well plates. The number of viable cells in each well was determined by adding 10 μL 5 mg/mL MTT solution. The cells were dissolved with 100 μL of solution that contained 20% SDS and 50% dimethyl formamide after cells had been incubated for 4 h at 37℃. The optical densities were quantified at a test wavelength of 570 nm and a reference wavelength of 630 nm using a Varioskan Flash Reader spectrophotometer (THERMO, MA, USA)[9].

Cell apoptosis analysis by fluorescence staining

The HCC cells were cultured in 6-well plates (3.0×10^5 cells/well) and treated with different concentrations of galangin at 37℃ in a humidified atmosphere with 5% CO_2 for 24 h. Cell apoptosis was evaluated by *in situ* uptake of propidium iodide and Hoechst 33258 as described by McKeague *et al*[10]. Briefly, galangin-treated cells were washed with phosphate buffered saline (PBS), and incubated in PBS containing 40 μg/mL propidium iodide and 2.5 μg/mL Hoechst 33258 for 10 min. Five hundred microliters of methanol: acetic acid (3: 1) fixative were then added directly to each well. Cells were viewed under fluorescence microscopy (Nikon Eclipse ET2000-E, Japan). The apoptotic index was calculated from the number of apoptotic nuclei *vs* total number of nuclei at each visual field.

Measurement of mitochondrial membrane potential[11]

HCC cells were treated with different concentrations of galangin for different times. Cells were then treated with rhodamine 123 with a final dye concentration of 10 μg/mL at 37℃ for 15 min, 5% CO_2 atmosphere prior to examination. Mitochondrial membrane potential was determined by flow cytometry. The change of fluorescent intensity of rhodamine 123 indicated the change in mitochondrial membrane potential.

Overexpression and knockdown of Bcl-2

The HCC cells were transfected with different plasmids [pcDNA3.1(+)-*Bcl-2*, pcDNA3.1(+)Vectors] using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol. Bcl-2 siRNA sequence was synthesized according to Fu's report[12], sense sequence: 5'-CGGAGGCUGGGAUGCCUUUdTdT-3', antisense sequence: 3'-dTdTGCCUCCGACCCUACGGAAA-5'. Before transfection, cells were seeded in 6 well plates or 60 mm tissue culture dishes containing DMEM medium without antibiotics for 24 h. Cells were transfected using lipofectamine 2000 with 2 μg plasmid or 100 pmol siRNA in each well. Bcl-2 protein level was measured by immu-

noblot analysis 24 h post-transfection. Cells were treated with galangin for another 24 h, and viability was determined by MTT and fluorescence staining methods.

Preparation of proteins in the mitochondrial and cytosolic fractions

Twenty four hours after treating with different concentrations of galangin, cells were washed twice in ice-cold PBS and resuspended in five volumes of ice-cold extract buffer (20 mmol/L Hepes-KOH, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, and 0.1 mmol/L PMSF, pH 7.5). The resuspended cells were homogenized and centrifuged at 750 *g* for 10 min at 4℃. The supernatants were centrifuged at 13000 *g* for 15 min at 4℃ to obtain the mitochondrial pellets. The remaining supernatants were centrifuged to obtain the cytosolic fractions. The protein concentrations of the resulting supernatants and mitochondrial fractions were measured.

Western blotting

The cells were loaded with cell decomposition buffer (pH 8.0) that contained 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% NP40, 0.05% phenylmethanesulfonyl fluoride (PMSF), 2 μg/mL aprotinin (Sigma, USA), and 2 μg/mL leupeptin (Sigma, USA). The proteins were determined as described previously by Western blotting[9] using the antibody (Santa Cruz Biotechology, Santa Cruz, CA, USA), and Western blotting luminal reagent (Amersham Biosciences, Uppsala, Sweden).

Statistical analysis

The values given are presented as mean ± SD. Statistical analysis was performed using one-way analysis of variance with LSD test. In all cases, $P < 0.05$ was considered as significant.

RESULTS

Galangin inhibits proliferation of HCC cells

We used the MTT assay to determine the effects of galangin on the proliferation of HCC cells. Using galangin at concentrations of 46.25, 92.5, 185 and 370 μmol/L, we observed an anti-proliferative effect on HCC cells that was dose-dependent (Figure 1A). Additionally, galangin could also inhibit HCC cell proliferation in a time-dependent manner (Figure 1B). The IC_{50} of galangin to HCC cells (HepG2, Hep3B, and PLC/PRF/5) 24 h post-treatment were 134.0, 87.3 and 79.8 μmol/L, respectively.

Galangin induces apoptosis of HCC cells

To determine whether galangin-treated HCC cells undergo apoptosis, we stained cells using Hoechst 33258/PI. As shown in Figure 2A, we observed a significant increase in the number of cells that exhibited nuclear condensation when treated with galangin for 24 h. This observation was similarly found in all three HCC cell lines tested. Our data also showed that the apoptotic index of the three HCC cells increased in a dose-dependent manner treated by galangin (Figure 2B).

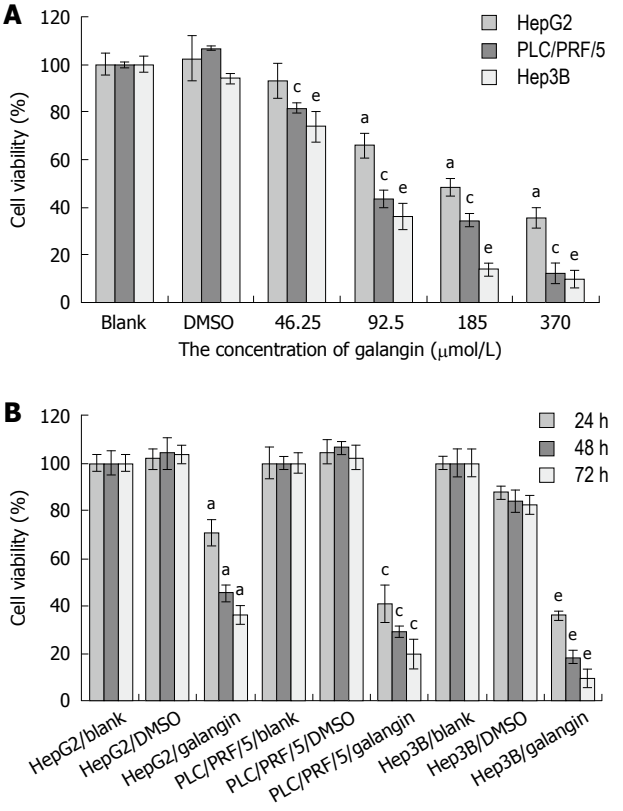


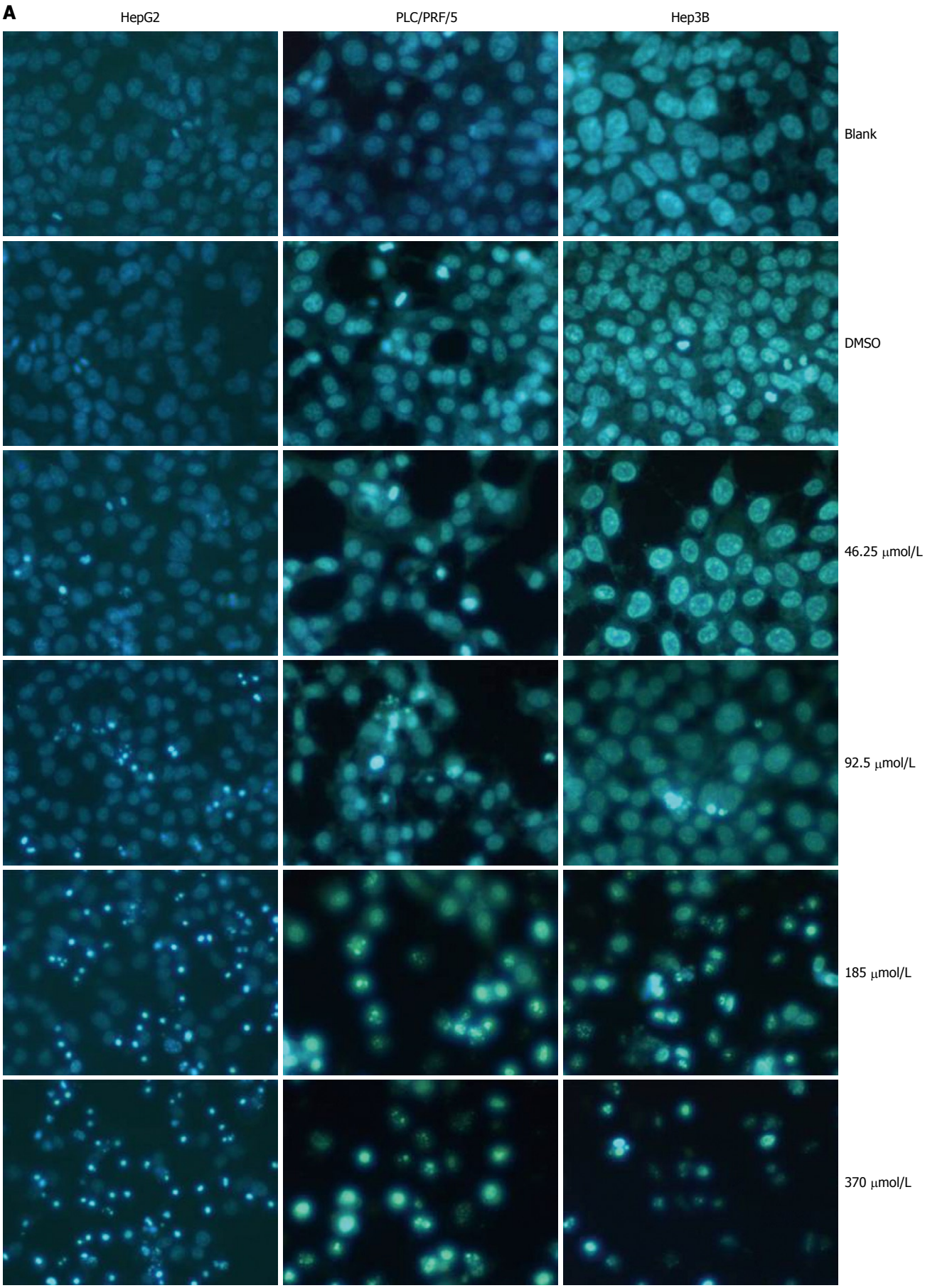
Figure 1 Effects of galangin on cell viability of three hepatocellular carcinoma cell lines. A: Three hepatocellular carcinoma (HCC) cell lines were treated with 46.25, 92.5, 185, and 370 μmol/L galangin for 24 h. The IC_{50} of galangin to HepG2, Hep3B, and PLC/PRF/5 cells were 134.0, 87.3 and 79.8 μmol/L, respectively; B: Three HCC cell lines were treated with 92.5 μmol/L galangin for 24, 48 and 72 h. mean ± SD. $n = 4$. $^aP < 0.05$ *vs* HepG2 cell/dimethyl sulfoxide (DMSO)-treatment group; $^cP < 0.05$ *vs* PLC/PRF/5 cell/DMSO-treatment group; $^eP < 0.05$ *vs* Hep3B/DMSO cell/DMSO-treatment group.

Galangin reduces the mitochondrial membrane potential of HCC cells

We observed a reduction in the mitochondrial membrane potential of all three HCC cell lines after treating with galangin (Figure 3). The mitochondrial membrane potential of the HCC cells decreased following treatment with galangin and this occurred in a dose- and time-dependent manner.

Galangin affects protein levels involved in the apoptosis pathway of HCC cells

In Figure 4, we evaluated the effects of galangin on protein expression involved in apoptosis of the HCC cell lines. Translocation of Bax to the mitochondria can alter the outer mitochondrial membrane permeability. Some pro-apoptotic proteins, including cytochrome c and AIF, are released into the cytosol from the mitochondria. Our data shows that Bax levels in the mitochondrial fraction of galangin-treated HCC cells increased in a dose-dependent manner, suggesting that the translocation of Bax into the mitochondria was involved in cell death induced by galangin (Figure 4A). Furthermore, we observed that the levels of cytochrome c and AIF in the cytosol of galangin-treated HCC cells increased in a dose-dependent manner. This suggests that the mitochondrial release of


A
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DMSO
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92.5 μmol/L
185 μmol/L
370 μmol/L

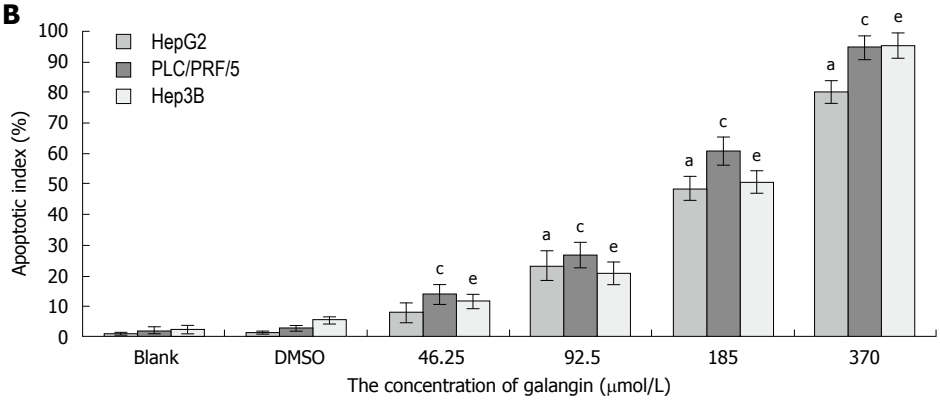

Figure 2 Hepatocellular carcinoma cells apoptosis induced by galangin. A: Morphology of apoptotic cells, pictures were taken under a 20 × objective; B: Cells were treated with different concentrations of galangin for 24 h and stained with Hoechst 33258/PI to measure apoptosis. mean ± SD. $n = 4$. $^{a}P < 0.05$ *vs* HepG2 cell/dimethyl sulfoxide (DMSO)-treatment group; $^{c}P < 0.05$ *vs* PLC/PRF/5 cell/DMSO-treatment group; $^{e}P < 0.05$ *vs* Hep3B/DMSO cell/DMSO-treatment group.

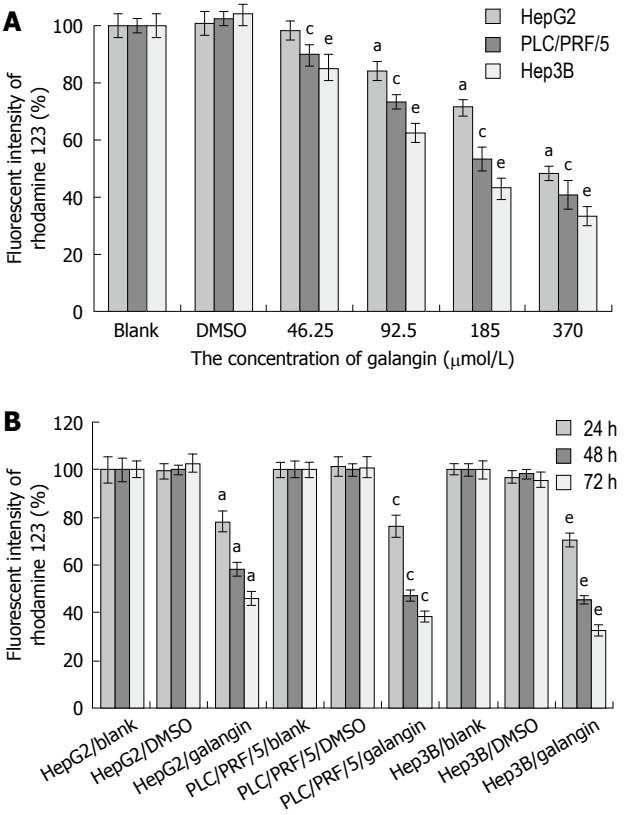

Figure 3 Effect of galangin on the mitochondrial membrane potential of hepatocellular carcinoma cell lines. A: Three hepatocellular carcinoma (HCC) lines were treated with 46.25, 92.5, 185, and 370 μmol/L galangin for 24 h; B: Three HCC were treated with 92.5 μmol/L galangin for 24, 48 and 72 h. mean ± SD. $n = 4$. $^{a}P < 0.05$ *vs* HepG2 cell/dimethyl sulfoxide (DMSO)-treatment group; $^{c}P < 0.05$ *vs* PLC/PRF/5 cell/DMSO-treatment group; $^{e}P < 0.05$ *vs* Hep3B/DMSO-treatment group.

cytochrome c and AIF into the cytosol may play a role in induction of cell apoptosis by galangin (Figure 4B). We also analyzed the effects of galangin on caspase-3 and caspase-9 activation, and poly(ADP-ribose) polymerase (PARP) cleavage by Western blotting. We showed caspase-9 activation in a dose-dependent manner, and observed a concomitant increase of caspase-3 activation and PARP cleavage (Figure 4C).

Bcl-2 protein level affects galangin-induced HepG2 cell apoptosis

To determine the role of Bcl-2 in galangin-treated HepG2 cells, we overexpressed and downregulated Bcl-2 by transfecting cells with pcDNA3.1-Bcl-2 and siRNA targeting Bcl-2, respectively. In Figure 5A, we show that we were able to overexpress and knockdown Bcl-2 sufficiently. As shown in Figure 5B and C, HepG2 cells overexpressing Bcl-2 were more resistant to galangin-induced apoptosis than control cells ($P < 0.05$). However, Bcl-2-knockdown HepG2 cells were more sensitive to galangin leading to decreased cell survival ($P < 0.05$) (Figure 5D). Taken together, these observations suggest that galangin induces apoptosis of HepG2 cell apoptosis *via* the mitochondrial pathway.

DISCUSSION

Studies have showed that most flavonoids exhibit anti-proliferative effects against tumor derived cell lines including leukemia[13], melanoma[14], colon[15], breast carcinoma[16], lung, and prostate[17]. Some reports have demonstrated that galangin is a naturally occurring non-toxic flavonoid with chemopreventive and anti-proliferative effects[6-8,18,19].

In this study, we demonstrated that galangin inhibited the proliferation of HCC cells and induced apoptosis at concentrations as low as 46.25 μmol/L and in excess of 185 μmol/L, respectively. Galangin-induced apoptosis was characterized by analyzing the effects on caspase-3 activation, PARP cleavage, and DNA condensation in HCC cells.

The mitochondrial pathway is commonly involved in the death stimuli. There are primarily two major events involved in apoptosis *via* the mitochondrial pathway. The first event is a change in mitochondrial membrane permeability, which leads to decreased mitochondrial membrane potential. Our data demonstrated reduced mitochondrial membrane potential as indicated by rhodamine 123 staining following treatment with galangin at different concentrations. The second event in the mitochondria-induced apoptotic pathway is the release of cytochrome c and AIF from the intermembrane space of the mitochondria into the cytosol. Here, we also showed that galangin increased

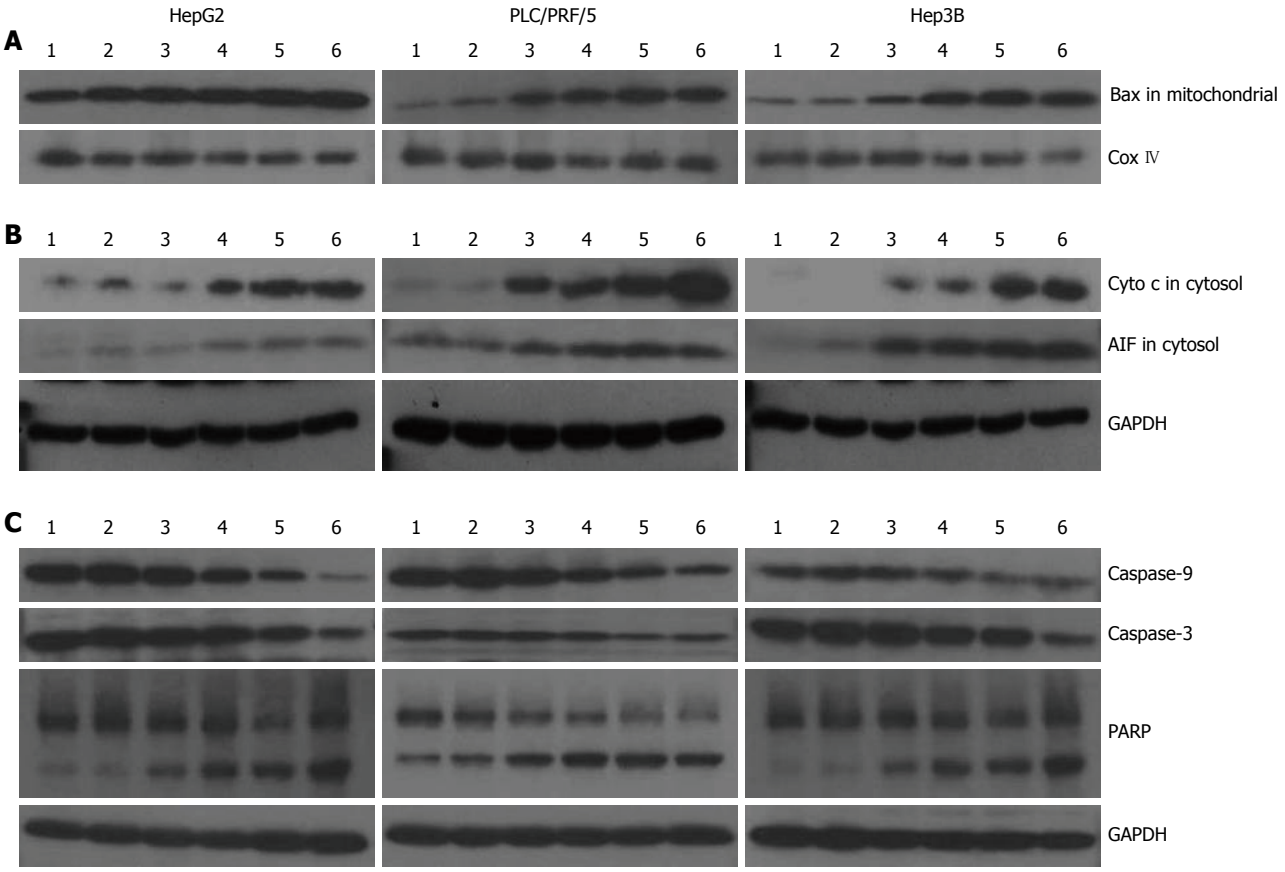

Figure 4 Effects of galangin-treated hepatocellular carcinoma cells on protein expression involved in the mitochondria pathway. A: The changes of Bax in the mitochondria; B: The changes of cytochrome c and apoptosis-inducing factor (AIF) in the cytosol; C: Caspase-3 and caspase-9 activation, and Poly(ADP-ribose) polymerase (PARP) cleavage induced by galangin. 1: Blank control group; 2: Dimethyl sulfoxide-treatment groups; 3: Cells were treated with 46.25 μmol/L galangin for 24 h; 4: Cells were treated with 92.5 μmol/L galangin for 24 h; 5: Cells were treated with 185 μmol/L galangin for 24 h; 6: Cells were treated with 370 μmol/L galangin for 24 h.

the release of cytochrome c and AIF in the cytosol. Thus, our data indicates that galangin-induced apoptosis of HCC cells occurs through the mitochondrial pathway.

The Bcl-2 family of proteins is involved in the mitochondrial apoptotic pathway by inducing the release of cytochrome c from the mitochondrial intermembrane space. Cytochrome c cooperates with Apaf-1 (Apoptotic protease activating factor 1) to induce caspase activation, leading to cell apoptosis[20]. The Bcl-2 family proteins are categorized into three groups based on the four Bcl-2 homology domains (BH1-4 domains). Bcl-2, Bcl-w, Bcl-xL and Mcl-1, which contain BH domains 1-4 and are localized to the outer mitochondrial membrane, are anti-apoptotic Bcl-2 proteins[21]. These proteins can directly bind and inhibit the proapoptotic Bcl-2 family in the mitochondria pathway of apoptosis. The proapoptotic proteins of Bcl-2 family members are functionally divided into two classes. One class is the effector molecule, which includes Bak and Bax, and permeabilizes the outer mitochondrial membrane to release cytochrome c into the cytosol. The other class is the BH3-only proteins including Bad, Bid, Bik, Bim, Bmf, bNip3, Hrk, Noxa and Puma, which promote cell apoptosis through protein-protein interactions with other Bcl-2 family members[21].

Our data indicates that galangin causes Bax translocation to the mitochondria in HCC cells. In non-apoptotic cells, Bax is located in the cytosol or loosely bound to the outer membrane of the mitochondria in monomeric forms. However, Bax translocates to the mitochondrial membrane and homodimerizes in the presence of a death signal. As a result, the outer mitochondrial membrane is permeable to release cytochrome c and AIF into the cytosol. The release of cytochrome c, which is an important protein in the electron transfer chain, can lead to reduced mitochondria membrane potential and adenosine triphosphate (ATP) synthesis. The results of our experiments showed that galangin induces cytochrome c release and decreases mitochondrial membrane potential.

In the cytosol, cytochrome c can bind to Apaf-1, which is a cytosolic protein. Apaf-1 undergoes a conformational change upon binding to dATP or ATP, leading to the formation of the apoptosome complex. The apoptosome recruits procaspase-9, resulting in caspase 9-caspase 3 activation. This caspase cascade is responsible for the hydrolysis of key cytoplasmic proteins and for the cleavage of genomic DNA nucleosomes into 180 bp fragments *via* caspase-activated DNase, such as PARP. Caspase-3 is an executioner of apoptosis that subsequently cleaves many important intracelluar substrates, leading to chromatin condensation, nucleosomal DNA fragmentation, nuclear membrane breakdown, externalization of phosphatidylserine, and formation of apoptotic bodies[22]. Our data also shows galangin-treated HCC cells did indeed cause caspase-9 and caspase-3 activation, and PARP cleavage.

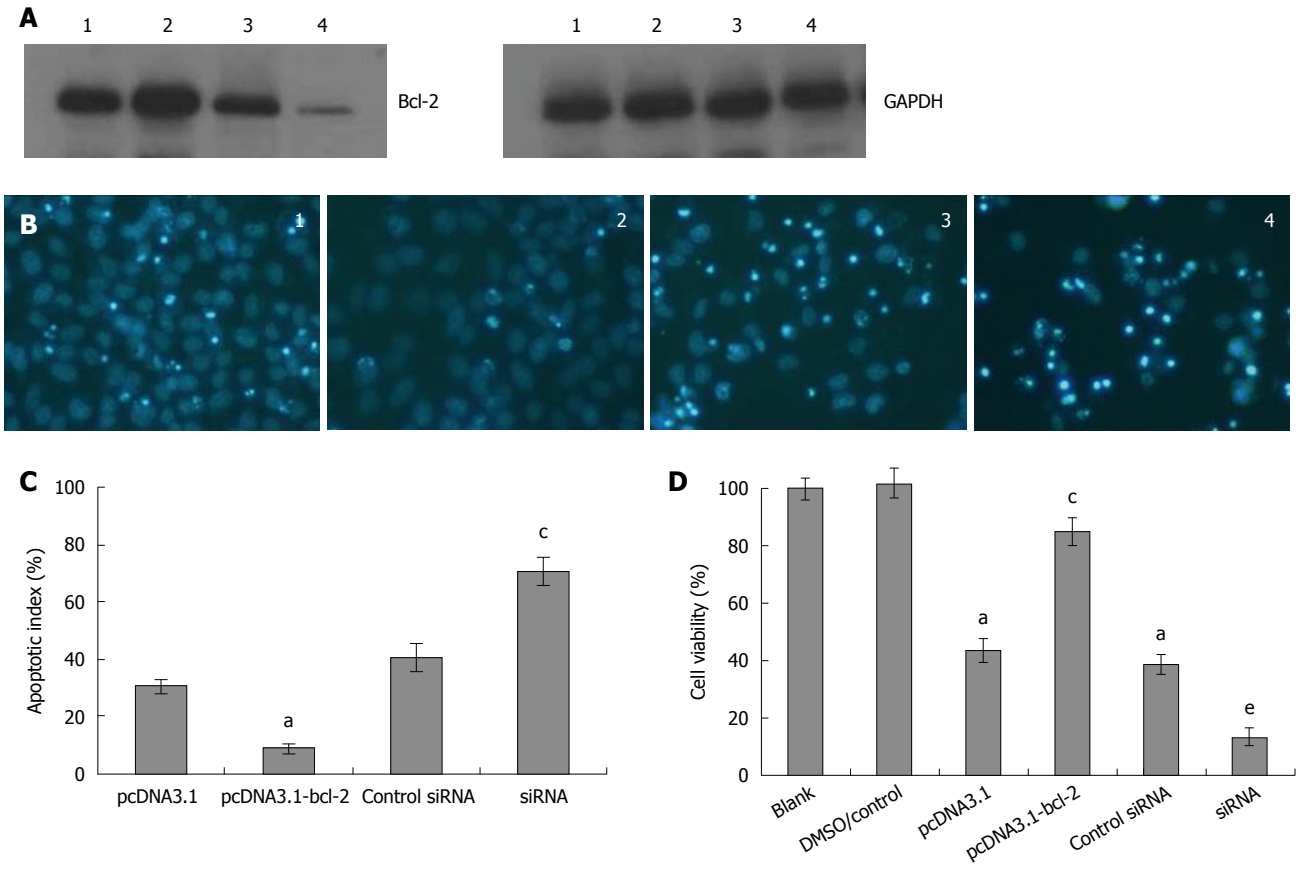

Figure 5 Effects of Bcl-2 on galangin-induced HepG2 cell apoptosis. A: Bcl-2 protein level was measured in HepG2 cells by Western blotting after cells were transfected; B: Morphology of apoptotic cells. Cells were treated with 185 μmol/L galangin for 24 h and stained with Hoechst 33258/PI to measure apoptosis. Pictures were taken under a 20 × objective. 1: Control vector group; 2: pcDNA3.1-bcl-2-transfected group; 3: Control siRNA group; 4: Bcl-2 siRNA-transfected group; C: Bcl-2 level affect apoptotic index of HepG2 cell induced by galangin. mean ± SD. $n = 4$. $^aP < 0.05$ *vs* pcDNA3.1 group; $^cP < 0.05$ *vs* control siRNA group; D: The effect of Bcl-2 level on cell viability induced by galangin. mean ± SD. $n = 4$. $^aP < 0.05$ *vs* HepG2 cell/dimethyl sulfoxide-treatment group; $^cP < 0.05$ *vs* pcDNA3.1-transfected group; $^eP < 0.05$ *vs* control siRNA group.

In our study, overexpression of Bcl-2 could suppress the apoptotic effects of galangin on HCC cells. Bcl-2 is an important anti-apoptotic protein that suppresses different drug-induced activation of the mitochondria-apoptotic pathway, such as etoposide[23], berberine[24], safatoposide[25], epigallocatechin-3-gallate[26], curcumin[27] and anti-inflammatory drugs[28]. The Bcl-2 protein can block the oligomerization of Bax and Bak and inhibit the apoptotic program[29,30]. Moreover, our data also showed that Bcl-2 decrease could enhance HCC cell sensitivity to galangin. These results show that Bcl-2 can modulate the effects of galangin on HCC cells and indicate that galangin induces apoptosis *via* the mitochondrial pathway.

We demonstrated that AIF is released from mitochondria into the cytosol in HCC cells upon galangin treatment. AIF migrates into the nucleus and induces high-molecular-mass DNA fragmentation and marginal chromatin condensation independent of caspases[31,32]. Therefore, HCC cells undergoing apoptosis may be due to a combination of caspase activation and AIF release.

In summary, we demonstrate that galangin induces HCC cell apoptosis *via* the mitochondrial pathway. Our data demonstrated that (1) galangin induces HCC cell apoptosis by triggering Bax translocation to the mitochondria; (2) galangin-treated HCC cells causes the release of AIF and cytochrome c into the cytosol from the mitochondria; and (3) overexpression of Bcl-2 attenuated galangin-induced HepG2 cells apoptosis, while down-regulated Bcl-2 expression enhanced galangin to induce cell apoptosis.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, particularly in China. However, HCC remains one of the more difficult cancers to treat. It is important to screen for new anti-cancer drugs. Dietary compounds possess anti-cancer properties.

Research frontiers

A number of dietary compounds possess anti-cancer properties. These dietary compounds may modify the activity of specific targets that control cell proliferation and apoptosis. Galangin could inhibit the methoxyresorufin *O*-demethylase activity of CYP1A2, CYP1A1 and P-form phenolsulfotransferase. Galangin induced apoptosis in several cancer cell lines and arrested the cell cycle, modulated the expression of cycline/cdk, and decreased Bcl-2. It was suggested that galangin may be a potential anti-tumor agent. However, the mechanism by which galangin exerts its anti-tumor activity is unknown.

Innovations and breakthroughs

In this study, the authors demonstrate the effects of galangin on HCC cells and elucidate the mechanism by which galangin induces apoptosis. This is the first study to report that galangin mediates apoptosis through a mitochondrial pathway. Galangin may be a potential chemotherapeutic drug for the treatment of hepatocellular carcinoma cells.

Applications

Understanding the mechanism by which galangin induces apoptosis may lead

to its use as an anti-cancer treatment of HCC. This study may represent a future potential chemotherapeutic drug in the treatment of HCC with galangin.

Terminology

The mitochondrial pathway is an important apoptotic pathway involved in the mitochondrial membrane permeability change and the release of cytochrome c and apoptosis-inducing factor (AIF) from the intermembrane space of the mitochondria into the cytosol. Bcl-2 is an important anti-apoptotic protein that suppresses different drug-induced activation of the mitochondria-apoptotic pathway.

Peer review

In this study, Zhang *et al* demonstrated that galangin, a polyphenolic compound from herbs, induced apoptosis in hepatocellular carcinoma cells through the mitochondrial pathway. Galangin treatment led to inhibition of cell growth, nuclear fragmentation, mitochondrial membrane potential collapse, caspase activation, Bax mitochondrial translocation, and release of cytochrome c and AIF. Modulation of Bcl-2 by overexpression or knockdown altered galangin-induced apoptosis. The results are convincing and the study is informative. The manuscript could be improved by making several changes.

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ORIGINAL ARTICLE

8-bromo-7-methoxychrysin-induced apoptosis of hepatocellular carcinoma cells involves ROS and JNK

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Abstract

AIM: To investigate whether the apoptotic activities of 8-bromo-7-methoxychrysin (BrMC) involve reactive oxygen species (ROS) generation and c-Jun N-terminal kinase (JNK) activation in human hepatocellular carcinoma cells (HCC).

METHODS: HepG2, Bel-7402 and L-02 cell lines were cultured *in vitro* and the apoptotic effects of BrMC were evaluated by flow cytometry (FCM) after propidium iodide (PI) staining, caspase-3 activity using enzyme-linked immunosorbent assay (ELISA), and DNA agarose gel electrophoresis. ROS production was evaluated by FCM after dichlorodihydrofluorescein diacetate (DCHF-DA) probe labeling. The phosphorylation level of JNK and c-Jun protein was analyzed by Western blotting.

RESULTS: FCM after PI staining showed a dose-dependent increase in the percentage of the sub-G1 cell population ($P < 0.05$), reaching 39.0% ± 2.8% of HepG2 cells after 48 h of treatment with BrMC at 10 μmol/L. The potency of BrMC to HepG2 and Bel-7402 (32.1% ± 2.6%) cells was found to be more effective than the lead compound, chrysin (16.2% ± 1.6% for HepG2 cells and 11.0% ± 1.3% for Bel-7402 cell) at 40 μmol/L and similar to 5-flurouracil (33.0% ± 2.1% for HepG2 cells and 29.3% ± 2.3% for Bel-7402 cells) at 10 μmol/L. BrMC had little effect on human embryo liver L-02 cells, with the percentage of sub-G1 cell population 5.4% ± 1.8%. Treatment of HepG2 cells with BrMC for 48 h also increased the levels of active caspase-3, in a concentration-dependent manner. z-DEVD-fmk, a caspase-3-specific inhibitor, prevented the activation of caspase-3. Treatment with BrMC at 10 μmol/L for 48 h resulted in the formation of a DNA ladder. Treatment of cells with BrMC (10 μmol/L) increased mean fluorescence intensity of DCHF-DA in HepG2 cells from 7.2 ± 1.12 at 0 h to 79.8 ± 3.9 at 3 h and 89.7 ± 4.7 at 6 h. BrMC did not affect ROS generation in L-02 cells. BrMC treatment failed to induce cell death and caspase-3 activation in HepG2 cells pretreated with N-acetylcysteine (10 mmol/L). In addition, in HepG2 cells treated with BrMC (2.5, 5.0, 10.0 μmol/L) for 12 h, JNK activation was observed. Peak JNK activation occurred at 12 h post-treatment and this activation persisted for up to 24 h. The expression of phosphorylated JNK and c-Jun protein after 12 h with BrMC-treated cells was inhibited by N-acetylcysteine and SP600125 pre-treatment, but GW9662 had no effect. SP600125 substantially reduced BrMC-induced cell death and caspase-3 activation of HepG2 cells. N-acetylcysteine and GW9662 also attenuated induction of cell death and caspase-3 activation in HepG2 cells treated with BrMC.

CONCLUSION: BrMC induces apoptosis of HCC cells by ROS generation and sustained JNK activation.

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Key words: Hepatocellular carcinoma; 8-bromo-7-methoxychysin; Chrysin; Reactive oxygen species; Jun N-terminal kinase

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INTRODUCTION

Epidemiological and intervention studies in both humans and animals have shown that regular consumption of fruits, vegetables, and tea is associated with decreased risk of cancer[1]. Fruits, vegetables, and tea provide essential nutrients and many diet-derived phenolics, in particular flavonoids, which have been demonstrated to exert potential anticarcinogenic activities[2]. Chrysin (5,7-dihydroxyflavone, ChR), a natural and biologically active flavone extracted from many plants, honey, and bee propolis, has been shown to inhibit cell proliferation and induce apoptotic cell death in a variety of cancer cells. Investigations into the molecular mechanisms underlying the inhibition of cell proliferation and induction of apoptosis by ChR have shown that ChR inhibited the growth by downregulating expression of proliferating cell nuclear antigen in HeLa cells[3], induced apoptosis through caspase activation and Akt inactivation in leukemia cells[4-7], and induced cell cycle arrest in human colon carcinoma cells, human esophageal adenocarcinoma OE33 cells and human lung adenocarcinoma cells[8-10]. Nevertheless, poor oral bioavailability has been a major limitation for the successful use of dietary flavonoids as cancer chemotherapeutic agents[11]. It has been reported that ChR halogenated derivatives had stronger bioactivities than the lead compound[12]. The higher hepatic metabolic stability and intestinal absorption of the methylated polyphenols make them more favorable than the unmethylated polyphenols for development as potential cancer chemopreventive agents[13]. Our previous study showed that the effect of 8-bromo-7-methoxychrysin (BrMC) on the inhibition of proliferation and induction of apoptosis in a colon cancer cell line, HT-29, and a gastric cancer cell line, SGC-7901, was stronger than that of ChR[14-17]. However, the molecular mechanisms of induced apoptosis in human hepatocellular carcinoma cells (HCC) by BrMC were not clear.

Although flavonoids are generally considered as antioxidants, they can also generate reactive oxygen species (ROS) depending on their structure and molecular environment[18]. A number of flavonoids exert direct and indirect pro-oxidant effects by inhibiting the mitochondrial respiratory chain complexes I by inducing glutathione (GSH) depletion[19-23]. In addition, Kachadourian *et al*[19-24] have reported that chrysin is a potent inducer of ROS generation and GSH depletion in A549, HL-60, and PC-3 cells. We here demonstrate that BrMC induces apoptosis of human HCC at least partly by promoting generation of ROS, and ROS-dependent sustained activation of c-Jun N-terminal kinase (JNK).

MATERIALS AND METHODS

Cell culture and reagents

Human HCC HepG2 [AFP(+), no tumorigenicity in immunosuppressed mice], Bel-7402 [AFP(+),with high frequency of tumorigenicity], and human embryo liver L-02 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 mg/L penicillin, and 100 mg/L streptomycin, and incubated in a humidified atmosphere of 5% CO_2 at 37℃. BrMC was synthesized as described previously[14]. ChR was purchased from the Sigma Chemical Co. (St Louis, MO, USA). BrMC and ChR were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 0.1% in media. N-acetylcysteine (NAC), and GW9662 were obtained from Sigma. Caspase-3 substrate N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA), and the caspase-3 specific inhibitor Z-Asp-Glu-Val-Asp-CH2F (Z-DEVD-fmk), were obtained from Calbiochem (La Jolla, CA, USA). Dichlorodihydrofuorescein diacetate (DCHF-DA) was from Molecular Probes Inc. (Eugene, OR, USA). Rabbit anti-human total JNK was from Cell Signaling Technology (Beverly, MA, USA); mouse anti-human phospho-JNK, phospho-c-Jun, total c-Jun and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were from Cell Signaling Technology. The commercial anti-hepatoma agent 5-flurouracil (5-FU) was obtained from Sigma Chemical Co. and was used as an apoptotic inducer positive control whereas 0.1% DMSO was used as a negative control.

Flow cytometry with propidium iodide staining

As previously described[25], cells were treated with serum-free medium for 24 h, followed by treatment with media containing different concentrations of test agents for 48 h. Cells were collected and prepared as a single cell suspension by mechanical blowing with phosphate-buffered saline (PBS), washed with cold PBS twice, fixed with 700 mL/L alcohol at 4℃ for 24 h, stained with propidium iodide (PI), and cell apoptosis was detected using flow cytometry (FCM; American BD Company, FACS 420).

DNA agarose gel electrophoresis

As previously described[26], cells were treated with serum-free medium for 24 h, followed by treatment with media containing different concentrations of test agents for 48 h. Cells were washed twice with PBS and DNA was extracted with Apoptotic DNA Ladder Detection Kit (Bodataike Company, Beijing) according to the manufacturer's in-

structions. The extracted DNA was kept at 4℃ overnight. Then 8.5 μL of DNA sample was mixed with 1.5 μL of 6 × buffer solution, electrophoresed on 20 g/L agarose gel containing ethidium bromide at 40 V, and observed through the DBT-08 gel image analysis system.

Caspase-3 activity assay

To evaluate caspase-3 activity, cell lysates were prepared after treatment with test agents. Assays were performed in 96-well microtiter plates by incubating 20 μg cell lysates in 100 μL reaction buffer (1% NP-40, 20 mmol/L Tris-HCl (pH 7.5), 137 mmol/L NaCl, 10% glycerol) containing the 5 μmol/L caspase-3 substrate (DEVD-pNA). Lysates were incubated at 37℃ for 2 h. Thereafter, the absorbance at 405 nm was measured with an enzyme-labeling instrument (ELX-800 type). In the caspase inhibitors assay, cells were pretreated with a caspase-3 specific inhibitor (10 μmol/L, Z-DEVD-fmk) for 1 h prior to addition of test agents.

Determination of ROS

Intracellular ROS accumulation was measured by FCM using the fluorescent probe DCHF-DA. Briefly, cells were incubated with 10 μmol/L of DCHF-DA for 30 min at 37℃ in the dark after treatment with various concentrations of test agents. After incubation, the cells were washed with PBS and analyzed within 30 min by FCM equipped with an air-cooled argon laser tuned to 488 nm. The specific fluorescence signals corresponding to DCHF-DA were collected with a 525 nm band pass filter. As a rule, 10000 cells were counted in each determination.

Western blotting analysis

As previously described[27], cells were collected, washed 3 times with PBS, lysed in cell lysate containing 0.1 mol/L NaCl, 0.01 mol/L Tris-Cl (pH 7.6), 0.001 mol/L EDTA (pH 8.0), 1 μg/mL aprotinin, 100 μg/mL PMSF, and then centrifuged at 13000 × *g* for 10 min at 4℃. Extracted protein sample (25 μg total protein/lane) was added in the same volume of sample buffer solution and subjected to denaturation at 100℃ for 10 min, then electrophoresed on 100 g/L or 60 g/L sodium dodecyl sulfate polyacrylamide gel electrophoresis at 100 mA for 3 h, and finally transferred onto polyvinylidene fluoride membrane (PVDF) (Millipore). The PVDF membrane was treated with Tris-Buffered Saline Tween-20 (TBST) containing 50 g/L skimmed milk at room temperature for 2 h, followed by incubation with the first antibodies phospho-JNK, total JNK, phospho-c-Jun, total c-Jun and β-actin (1:1000 dilution), respectively, at 37℃ for 2 h. After being washed with TBST for 30 min, the corresponding secondary antibody was added and incubated at room temperature for 1 h. Bound antibody was visualized using chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, USA). Total JNK, total c-Jun and β-actin (1:1000 dilution) were used as an internal control. Images were scanned, followed by densitometry analysis with UN-SCAN-IT software (Silk Scientific).

Statistical analysis

The database was set up with the SPSS 15.0 software package (SPSS Inc., Chicago, IL, USA) for analysis. Data were represented as mean ± SD. The means of multiple groups were compared with one-way analysis of variance, after the equal check of variance, and two-two comparisons of the means were performed using the least significant difference method. Statistical comparison was also performed with two-tailed *t*-test when appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of BrMC on apoptosis in human HCC

To determine whether BrMC selectively induces apoptosis of human HCC, the human HCC lines HepG2 and Bel-7402 and human embryo liver L-02 cells were treated with increasing concentrations of BrMC for 48 h. Apoptotic cell death was examined by: (1) cell population with sub-G1 contents of DNA using FCM after PI staining; (2) caspase-3 activity determined by enzyme-linked immunosorbent assay; and (3) DNA fragmentation observed by DNA agarose gel electrophoresis. Figure 1A shows that there is a dose-dependent increase in the percentage of sub-G1 cell population ($P < 0.05$), reaching 39.0% ± 2.8% of HepG2 cells after 48 h of treatment with BrMC at 10 μmol/L. The potency of BrMC in HepG2 and Bel-7402 (32.1% ± 2.6%) cells was found to be more effective than the lead compound, chrysin (ChR, 16.2% ± 1.6% for HepG2 cells and 11.0% ± 1.3% for Bel-7402 cells) at 40 μmol/L and similar to 5-FU (33.0% ± 2.1% in HepG2 cells and 29.3% ± 2.3% in Bel-7402 cells) at 10 μmol/L. BrMC had little effect in human embryo liver L-02 cells, with the percentage of the sub-G1 cell population 5.4% ± 1.8%. Compared with HepG2 cells, Bel-7402 cells were less sensitive to BrMC. Parallel to the cell lethal effect and the enhanced caspase-3 activity, treatment of HepG2 cells with BrMC for 48 h increased the levels of active caspase-3, in a concentration-dependent manner (Figure 1B). Requirement of caspase activity for BrMC-induced apoptosis was examined using a caspase-3-specific inhibitor, z-DEVD-fmk. The data showed that z-DEVD-fmk was able to prevent activation of caspase-3 (Figure 1B). Similarly, treatment with BrMC at 10 μmol/L for 48 h resulted in the formation of a DNA ladder (Figure 2). These results indicate that BrMC selectively induced apoptotic cell death of HCC in a caspase-dependent fashion.

Effects of BrMC on ROS generation in HepG2 cells

Because oxidative damage plays an important role in anticancer effects of ChR[19], we subsequently examined the level of intracellular ROS in HepG2 and L-02 cells after treatment with BrMC using an oxidation-sensitive fluorescent probe DCHF-DA, which is oxidized to 2',7'-dichlorofluorescein (DCF) in the presence of ROS. Figure 3A shows that treatment of cells with BrMC (10 μmol/L) increased mean fluorescence intensity of DCF in HepG2 cell from 7.2 ± 1.12 at 0 h to 79.8 ± 3.9 at 3 h and 89.7 ±

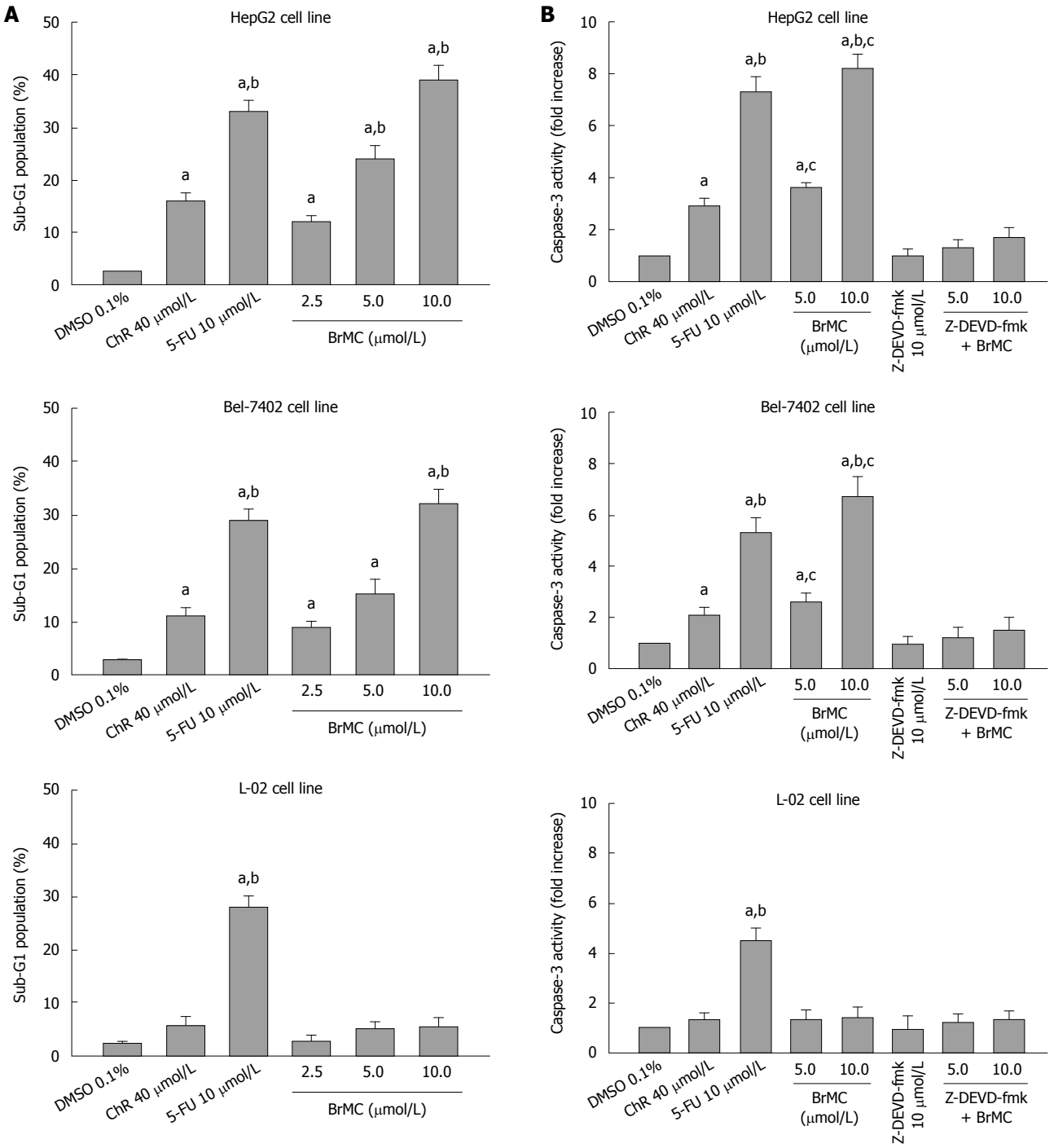

Figure 1 Effects of 8-bromo-7-methoxychrysin on the percentage sub-G1 cell population (A), caspase-3 activity (B) in human hepatocellular carcinoma HepG2, Bel-7402 and human embryo liver L-02 cells. $^{a}P < 0.05$ *vs* treatment with dimethyl sulfoxide (DMSO); $^{b}P < 0.05$ *vs* treatment with chrysin (ChR); $^{c}P < 0.05$ *vs* treatment with Z-Asp-Glu-Val-Asp-CH2F (Z-DEVD-fmk) plus BrMC. 5-FU: 5-flurouracil.

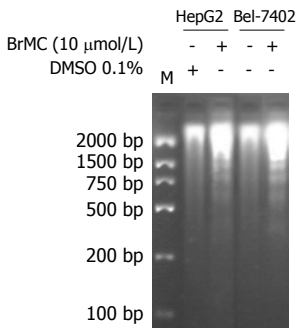

Figure 2 Effects of 8-bromo-7-methoxychrysin on DNA fragmentation in human hepatocellular carcinoma HepG2 and Bel-7402 cells. DMSO: Dimethyl sulfoxide.

4.7 for 6 h. However, BrMC did not affect ROS generation in L-02 cells. BrMC treatment failed to induce ROS generation in HepG2 cells pretreated with 10 mmol/L NAC. We next investigated whether generation of ROS induced by BrMC was accompanied by apoptotic cell death after BrMC treatment. To determine a link between elevation of the intracellular ROS level and apoptotic cell death in BrMC-treated cells, HepG2 cells were pre-incubated with the thiol-containing antioxidant NAC before treatment with BrMC. BrMC treatment failed to induce

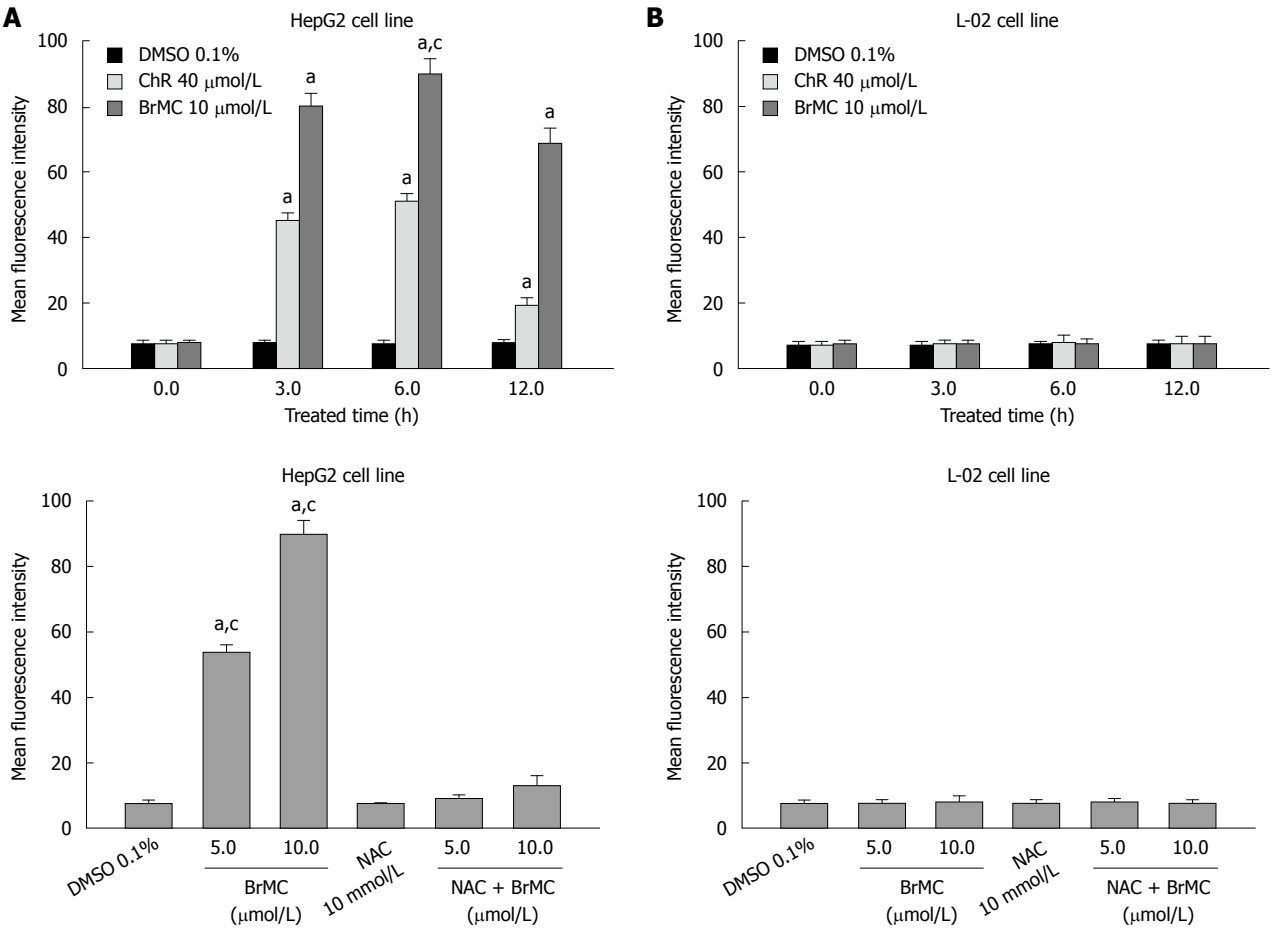

Figure 3 Effects of 8-bromo-7-methoxychrysin on reactive oxygen species generation in human hepatocellular carcinoma HepG2 (A) and human embryo liver L-02 cells (B). [a]*P* < 0.05 *vs* baseline or treatment with dimethyl sulfoxide (DMSO); [c]*P* < 0.05 *vs* treatment with N-acetylcysteine (NAC) plus 8-bromo-7-methoxychrysin (BrMC).

cell death and caspase-3 activation in cells pretreated with 10 mmol/L NAC (Figure 4). These observations suggest that a selective increase in the intracellular ROS level after BrMC treatment in HCC is required in the cell death pathway, accompanied by activation of caspase-3.

Effects of BrMC on JNK activation in HepG2 cells

It is well known that many therapeutic agents trigger apoptosis *via* activation of stress-related signaling pathways including JNK-mediated ones[28]. JNK plays distinct roles in cell death. Transient activation of JNK is believed to be antiapoptotic whereas persistent activation is pro-apoptotic[29,30]. Here we examined the effect of BrMC-induced JNK activation. JNK activation was measured by Western blotting analysis of phosphorylated JNK and its downstream target c-Jun. In HepG2 cells treated with BrMC (2.5, 5.0, 10.0 μmol/L) for 12 h, JNK activation was observed (Figure 5A). Time course experiments revealed peak JNK activation at 12 h post-treatment and this activation persisted for up to 24 h (Figure 5B).

Effects of BrMC-stimulated JNK activation on induction of apoptosis and caspase-3 activation in HepG2 cells

It has been reported that ChR derivatives induce apoptosis of human HCC and human gastric cancer cells by activating peroxisome proliferator-activated receptor-γ (PPARγ)[17,26]. One of the important components in ROS signaling is JNK activation[31]. These results prompted us to investigate whether NAC, an antioxidant, and GW9662, a blocker of PPARγ, and JNK inhibitor SP600125 affected the phosphorylated JNK protein level in HepG2 cells treated with BrMC. Figure 6A shows that the expression of phosphorylated JNK protein after 12 h with BrMC-treated cells was inhibited by NAC and SP600125 pretreatment, but GW9662 had no effect. To examine the effects of BrMC-stimulated JNK activation on induction of apoptosis and caspase-3 activation in HepG2 cells, we used the JNK inhibitor SP600125 to investigate the role of JNK in BrMC-induced cell death and caspase-3 activation. SP600125 substantially reduced BrMC-induced cell death and caspase-3 activation of HepG2 cells (Figure 6B-D). In addition, NAC and GW9662 also inhibited induction of cell death and caspase-3 activation in HepG2 cells treated with BrMC (Figure 6B-D). These results suggest that ROS production and JNK activation are required for BrMC-induced cell death and caspase-3 activation in HepG2 cells.

DISCUSSION

Our previous study showed that the effect of BrMC on

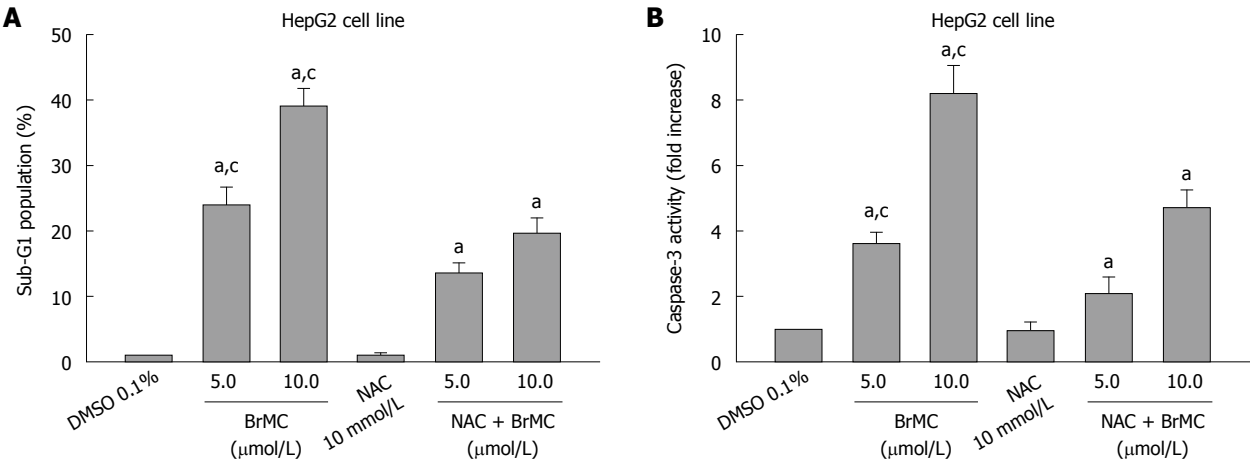

Figure 4 Effects of N-acetylcysteine on 8-bromo-7-methoxychrysin-induced apoptosis rate (A) and caspase-3 activity (B) in HepG2 cells. $^{a}P < 0.05$ *vs* treatment with medium (0 h) or dimethyl sulfoxide (DMSO); $^{c}P < 0.05$ *vs* treatment with N-acetylcysteine (NAC) plus 8-bromo-7-methoxychrysin (BrMC).

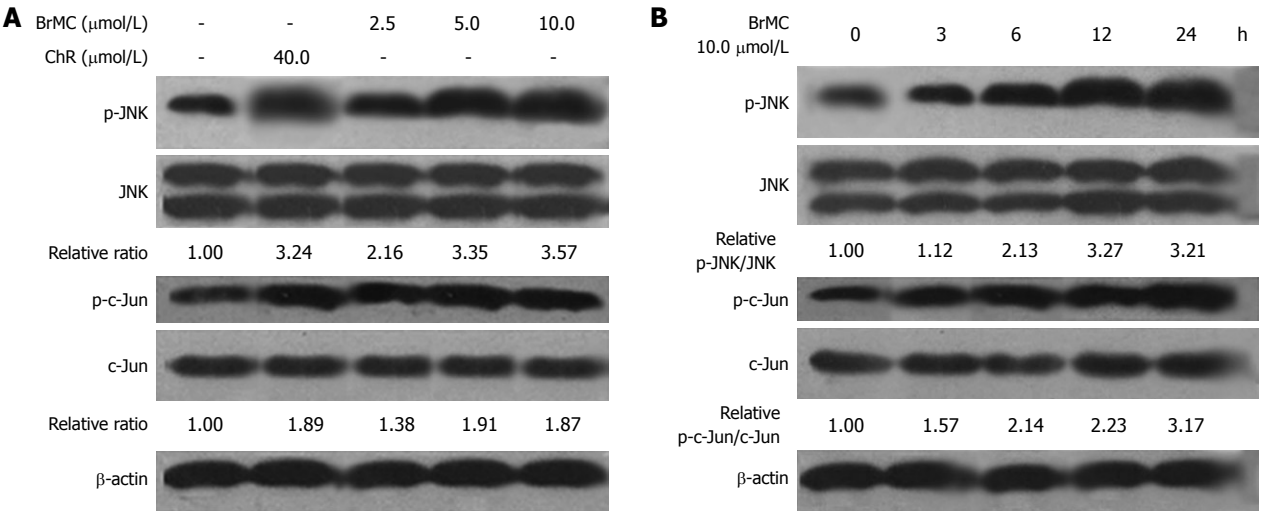

Figure 5 Effects of 8-bromo-7-methoxychrysin on the level of phosphorylated Jun N-terminal kinase and phosphorylated c-Jun in HepG2 cells (Western blotting, mean ± SD, *n* = 3). 8-bromo-7-methoxychrysin (BrMC) elevated the level of phosphorylated Jun N-terminal kinase (JNK) and phosphorylated c-Jun in a concentration-dependent manner (A) and in a time-dependent manner (B). The ratio of p-JNK/JNK or p-c-Jun/c-Jun was normalized to 0 h or the untreated group.

the inhibition of proliferation and induction of apoptosis in a colon cancer cell line HT-29 and gastric cancer cell line SGC-7901 was stronger than that of ChR[14,16]. In addition, it has been reported that ChR is a potent inducer of ROS generation and GSH depletion in A549, HL-60, and PC-3 cells[19,20]. In this study, we firstly showed that BrMC selectively induced apoptotic cell death of human HCC in a caspase-dependent fashion, with little effect on human embryo liver L-02 cells (Figures 1 and 2). The potency of BrMC in HepG2 and Bel-7402 cells was found to be greater than ChR and similar to 5-FU. Secondly, we indicated that BrMC selectively induced apoptosis of HepG2 cells and was accompanied by ROS generation. However, BrMC did not affect ROS generation of L-02 cells (Figures 3 and 4). Furthermore, we demonstrated that BrMC induced sustained activation of JNK in HepG2 cells in a ROS-dependent manner (Figures 5 and 6).

ROS have been associated with carcinogenesis but also, paradoxically, with mitochondrial-mediated cell death in cancer cells. The overproduction of ROS as a central event in mitochondrial-mediated apoptosis is now well documented[32-35]. The antioxidant properties of flavonoids have been associated with their cardioprotective and neuroprotective properties, yet such an association is much less certain concerning their cancer preventive properties. In the case of flavonoids, however, their chemopreventive properties may rather rely on eliminating precancerous cells because of their prooxidant properties *in vivo*. This is likely the case of apigenin and ChR, where their cytotoxicity may result from a combination of interference with the mitochondrial respiratory chain and multidrug resistance protein-mediated GSH depletion[36,37]. It is worth noting that the bee product propolis, which is known to exert antimicrobial, antiviral, and cancer preventive properties, contains ChR, a poor antioxidant, as one of its major components[38]. Intracellular ROS mediate multiple cellular responses, including protein kinase activation[39], cell cycle progression[40], myeloid cell differentiation[41,42], and apoptotic and necrotic cell death[43]. It has been reported in several studies that depletion of intracellular GSH plays

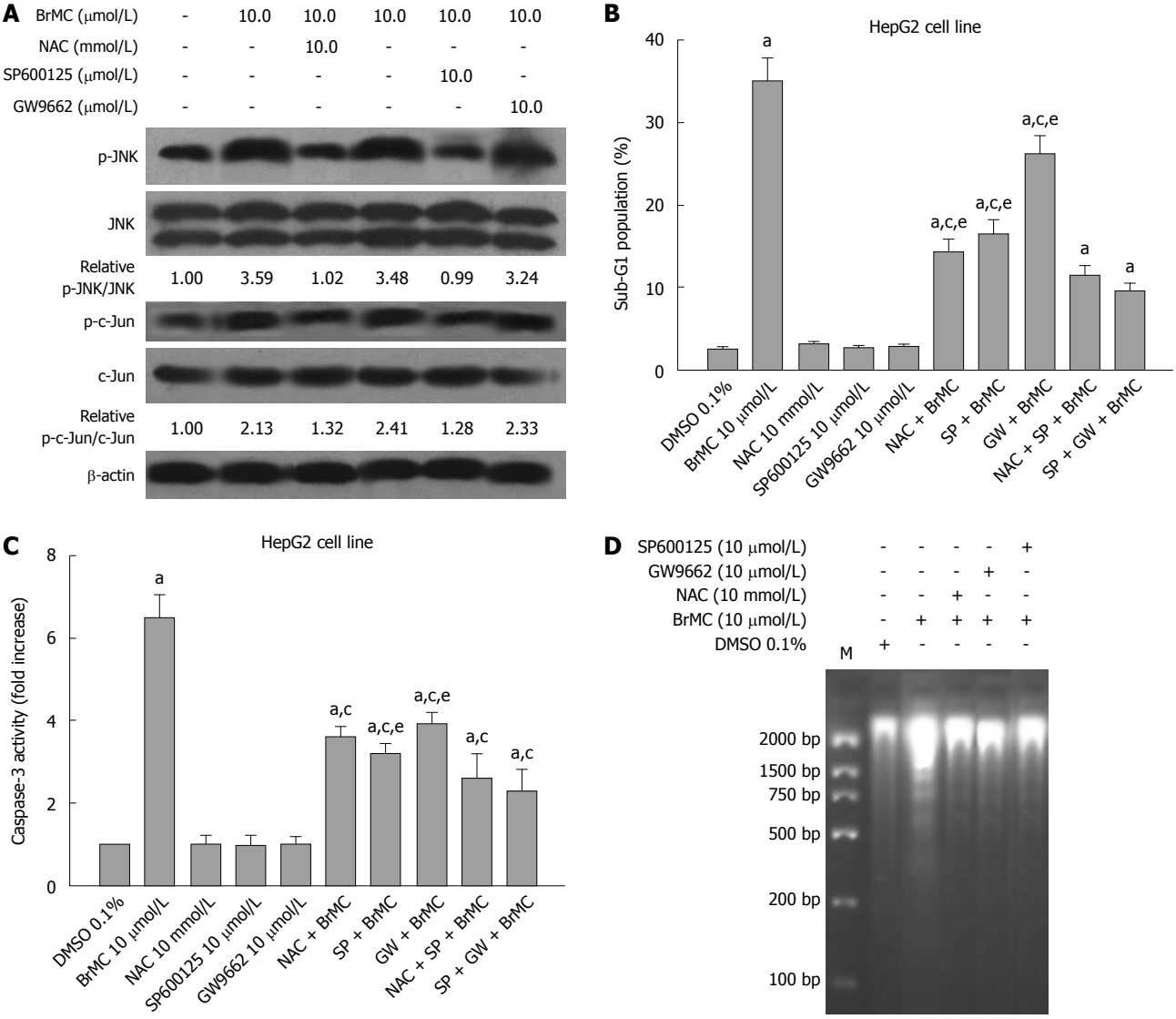

Figure 6 Effect of N-acetylcysteine, an antioxidant, and GW9662, a blocker of peroxisome proliferator-activated receptor-γ, and Jun N-terminal kinase inhibitor SP600125 on 8-bromo-7-methoxychrysin-induced activation of Jun N-terminal kinase (A), apoptosis (B), activation of caspase-3 (C), and DNA fragmentation (D) in HepG2 cells. The ratio of p-Jun N-terminal kinase (JNK)/JNK or p-c-Jun/c-Jun was normalized to 0 h or the untreated group. $^{a}P < 0.05$ *vs* treatment with dimethyl sulfoxide (DMSO); $^{c}P < 0.05$ *vs* treatment with 8-bromo-7-methoxychrysin (BrMC) alone; $^{e}P < 0.05$ *vs* treatment with N-acetylcysteine (NAC) in combination with SP600125 and BrMC or GW9662 in combination with SP600125 and BrMC.

a critical role in initiating apoptosis by ChR[10,19]. This is likely caused by the reversible interaction between ChR and GSH. In present study, we found that the ChR derivative, BrMC promoted accumulation of ROS products in a concentration-dependent manner in HepG2 cells but not in L-02 cells (Figure 3). NAC is an antioxidant agent and mainly known as a ROS scavenger. It reduces ROS generation and protects the cells from oxidative stress. BrMC-induced apoptosis of HepG2 cells was accompanied by ROS generation. It has been reported that arsenic trioxide-induced ROS generation was inhibited by NAC treatment[44]. We used NAC as an antioxidant to investigate the ROS generation induced by BrMC. NAC treatment not only reduced ROS generation but also attenuated induction of apoptosis in HepG2 cells (Figures 3 and 4). All together, these results indicate that induction of ROS generation contributes to BrMC-induced apoptosis in HepG2 cell line.

In this study, we investigated the signaling pathways affected by BrMC in HepG2 cells. We show that BrMC persistently activates JNK and induces apoptosis of HepG2 cells (Figures 5 and 6). BrMC-activated signaling pathways appear to activate executioner caspases because caspase 3 activity was enhanced in cells exposed to BrMC (Figure 1). JNK is implicated in mediating endoplasmic reticulum stress-induced apoptosis[45]. It has been shown that triterpenoids activate JNK leading to apoptosis[46,47]. In the present study, we detected both JNK activation and apoptotic cell death in cells exposed to BrMC. In addition, the presence of the JNK inhibitor SP600125 attenuated BrMC-induced apoptosis of HepG2 cells, indicating that BrMC-induced apoptosis is also JNK dependent (Figures 5 and 6). Collectively, we conclude that JNK activation mediates BrMC-induced apoptosis in the HepG2 cell line. We noted that SP600125 inhibited BrMC-induced c-Jun phosphorylation completely, but only partially prevented induction of apop-

tosis by BrMC in HepG2 cells (Figure 5). Thus, we cannot role out the possibility that other mechanisms also participate in BrMC-induced apoptosis in the HepG2 cell line.

It has been documented in several studies that depletion of intracellular GSH plays a critical role in initiating apoptosis by chrysin[10,19]. Zou *et al*[46]recently showed that triterpenoids deplete intracellular GSH, resulting in JNK-dependent apoptosis in human lung cancer A549 cells. In this study, we found that the presence of NAC blocked the effects of BrMC not only in generating ROS but also in activating JNK and triggering apoptotic cell death (Figure 6).

In summary, the present study has shown that BrMC promotes accumulation of intracellular ROS, resulting in sustained activation of JNK, leading to apoptosis in human HCC but not in human embryo liver L-02 cells. While further investigation is required to provide evidence for the efficacy of this HCC therapy in a nude mouse model and whether it reaches an effective dose *in vivo*, these results highlight a new mechanism responsible for BrMC-induced apoptosis, and raise the possibility that BrMC may be promising as a candidate for human HCC therapy.

COMMENTS

Background

Chrysin (5,7-dihydroxyflavone), a natural and biologically active flavone extracted from many plants, honey, and propolis, has been shown to inhibit cell proliferation and induce apoptotic cell death in a variety of cancer cells. Nevertheless, poor oral bioavailability has been a major limitation for the successful use of dietary flavonoids as cancer chemopreventative agents. The authors have synthesized 8-bromo-7-methoxyhysin (BrMC), a novel chrysin analogue. BrMC has been demonstrated to inhibit proliferation and induction of apoptosis in a colon cancer cell line HT-29 and gastric cancer cell line SGC-7901 and its effect was stronger than that of chrysin.

Research frontiers

Epidemiological and intervention studies in both humans and animals have shown that regular consumption of fruits, vegetables, and tea is associated with decreased risk of cancer. Fruits, vegetables, and tea provide essential nutrients and many diet-derived phenolics, in particular flavonoids, which have been demonstrated to exert potential anticarcinogenic activities. Flavonoids are plant polyphenolic compounds, which comprise several classes including flavonols, flavanones, flavanols, and flavans. Chrysin is a natural flavonoid contained in many plant extracts, honey, and propolis. Several studies in recent years have shown that chrysin and its derivatives have multiple biological activities, such as anti-inflammatory, anti-cancer, and anti-oxidative effects. However, the cellular and molecular mechanisms underlying chrysin and derivatives induced apoptosis of cancer cells are not clearly understood.

Innovations and breakthroughs

The authors firstly showed that BrMC, a novel chrysin analogue induced apoptotic cell death of human hepatocellular carcinoma cells (HCC) in a caspase-dependent fashion. BrMC-induced apoptosis of HepG2 cells was accompanied by ROS generation. Induction of reactive oxygen species (ROS) generation contributes to BrMC-induced apoptosis in a HepG2 cell line. In addition, they demonstrated that BrMC induced sustained activation of Jun N-terminal kinase (JNK) in HepG2 cells in a ROS-dependent manner.

Applications

The present study has shown that BrMC promotes accumulation of intracellular ROS, resulting in sustained activation of JNK, leading to apoptosis in human HCC. These results suggest that BrMC is a promising candidate for human HCC therapy.

Peer review

This is an original article by Jian-Guo Cao's group that investigated the effect of BrMC on HCC. They have found that BrMC induces apoptosis in HepG2 and Bel-7402 cells by generation of ROS, JNK activation, and activation of caspase-3. Overall the experiments were conducted appropriately, and the content is interesting.

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ORIGINAL ARTICLE

Effects of four *Bifidobacteria* on obesity in high-fat diet induced rats

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Abstract

AIM: To compare the effects of four *Bifidobacteria* strains (*Bifidobacteria L66-5*, *L75-4*, *M13-4* and *FS31-12*, originated from normal human intestines) on weight gain, lipid metabolism, glucose metabolism in an obese murine model induced by high-fat diet.

METHODS: Forty-eight Sprague-Dawley rats were randomly divided into six groups. Control group received standard chow, model group received high-fat diet, and intervention groups received high-fat diet added with different *Bifidobacteria* strains isolated from healthy volunteers' fresh feces. All rats were executed at the 6th weekend. Body weight (BW), obese indexes, oral glucose tolerance test, serum and liver lipid and serum insulin (INS) were tested. Liver lipid deposition was classified pathologically.

RESULTS: Compared with the model group, *B. M13-4* improved BW gains (264.27 ± 26.91 *vs* 212.55 ± 18.54, $P = 0.001$) while *B. L66-5* induced a decrease in BW (188.47 ± 11.96 *vs* 212.55 ± 18.54, $P = 0.043$). The rest two strains had no significant change in BW. All the four strains can reduce serum and liver triglyceride and significantly alleviate the lipid deposition in liver. All strains showed a trend of lowing serum and liver total cholesterol while *B. L66-5* and *B. FS31-12* did so more significantly. In addition, all the four strains showed no significant differences in serum INS and glucose level.

CONCLUSION: The response of energy metabolism to administration of *Bifidobacteria* is strain dependent. Different strains of *Bifidobacteria* might drive different directions of fat distribution.

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Key words: *Bifidobacterium*; Obesity; Serum lipid; Body weight

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INTRODUCTION

Obesity is becoming a global epidemic, and a major contributor to increased incidence of serious chronic diseases such as type 2 diabetes, cardiovascular diseases, hepatic and skeletal muscle insulin resistance, and certain forms of cancer[1]. Several strategies have been used to

treat obesity, including diet control, exercise, behavior therapy, medications and surgery. However, the diet control and exercise are too hard to be strictly carried on. Also, undesirable side effects of drugs have restricted their therapeutic use. Fortunately, some recent interesting researches on obesity are helping to validate a new approach to control this medical disorder.

Recent researches have demonstrated that obesity may lead to the composition shift of gut microbiota in both mice and humans. Fewer *Bacteroidetes* and more *Firmicutes* are colonized in the gut of obese people and animals, shown by some 16S-rRNA-gene-sequence-based comparative surveys of gut bacteria[2,3]. Weight loss makes the ratio of *Bacteroidetes* to *Firmicutes* up-regulated in humans[3]. Dietary inclusion of *Lactobacillus*, which belongs to *Bacteroidetes* and/or *Bifidobacterium*, can improve obesity both in murine model and humans[4-6]. The intentional manipulation of community structure of gut microbiota may be a novel strategy to treat obesity.

Bifidobacterium is one of the most numerous "probiotic" in mammalian gut among commensal bacteria, which belongs to *Actinomycetes* and also a kind of lactic acid bacteria. It can help *Bacteroides* degrade polysaccharides[7] and inhibit exogenous cholesterol absorption from the small intestine[8]. Although most researches focus on the hypocholesteremia effect of *Lactobacillus*[4,9-11], a study showed that a strain of *Bifidobacterium longum* exhibited a more significant effect in lowering serum total cholesterol than a mixed culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp.*bulgaricus* (SL) both in rats and humans[6]. In contrast, probiotics VSL#3 (a combination of *Streptococcus thermophilus* and several species of *Lactobacillus* and *Bifidobacteria*) was found to increase liver fat with no significant changes in comprehensive metabolic panel or in body weight (BW) in a clinical research[12]. These results suggested that different strains may have their specificities in colonization or function. Furthermore, *Bifidobacterium longum* can induce an expansion of *Bacteroides. Thetaiotaomicron*'s substrates range under co-colonized condition, while another *Bifidobacterium* species, *B. animalis* showed no significant impact[7]. *Bifidobacteria* preparations are safe, widely-used, and well-tolerant. Thus, some specific strains of *Bifidobacteria* related to lipid metabolism and BW may be a potential therapeutic candidate for management of obesity.

In order to screen out more efficient strains in obese management among different strains of *Bifidobacteria* originated from normal human intestines, we established an obese model in rats induced by high-fat diet, and compare their effects on weight gain, lipid metabolism and glucose metabolism. Several strains of *Bifidobacteria* showed dependant effects in obese control in this study, thus would act as potential therapeutic candidates.

MATERIALS AND METHODS

Preparation of bacterial cultures

Four *Bifidobacteria* strains were isolated from healthy volunteers' fresh feces in our facility, and identified according to biochemical characteristics (API 20A biochemical strip, BioMerieux sa). The four strains were named *B. L66-5*, *B. L75-4*, *B. M13-4* and *B. FS31-12*, respectively, and maintained at -80℃ in our laboratory. The strains were all grown in MRS medium under anaerobic condition. When measured at 600 nm (A_{600}), the exponential and stationary growth reached an optical density of 1-2 and 1-4.5, respectively. The correspondence between absorbance and bacterial counts was established (1 mL of culture at A_{600} = 1 contains about 10^8 colony-forming units, CFU). The number of CFU administered was routinely verified by plating. Strains were harvested by centrifugation at 2000 × *g* for 20 min, washed twice with neutral saline, and resuspended at 1 × 10^8 CFU/mL concentration in neutral saline, and 0.4 mL bacterial solution was administered to each rat by intragastric gavage.

Animals and diet

Forty-eight 3-wk-old male Sprague-Dawley (SD) rats were purchased from Slaccas Lab Animal Ltd, Shanghai, China, weighing 50-70 g. The animals were housed in individual stainless steel cages under standard conditions (20-22℃, 50%-55% humidity, 12/12 h dark/light cycle). The rats were fed with a solid standard chow [DongChuang Lab Animal Ltd., Hunan, China, including 17.53% (wt/wt) protein, 6.08% (wt/wt) fat, and 59.98% (wt/wt) carbohydrate, calories (1250 kj/100 g)] for 1 wk. After this adaptation period, 48 rats were randomly assigned to six dietary treatment groups, 8 rats in each group. Each rat was fed diet 13 g/d from the 1st wk, which was then increased by 2 g/d per week. Control group was fed on a standard chow, and the other groups were fed on a high-fat diet (HFD)(DongChuang Lab Animal Ltd, HuNan, China, 16.52% (wt/wt) protein, 25.17% (wt/wt) fat, and 56.66% (wt/wt) carbohydrate, calories (1810 kj/100 g). Each interventional group was administered with *B. L66-5*, *B. L75-4*, *B. M13-4* and *B. FS31-12*, respectively. Model and control groups were given equivalently 0.9% saline. All rats had free access to water and were supplied with bacteria liquid or 0.9% saline by intragastric gavage at a fixed time every day. The assigned diets were given to the rats for 6 wk. BW was measured weekly and caloric intake was accounted finally. All the rats were sacrificed by ether for further studies. The care and use of animals followed our institutional and national guidelines and all experimental procedures involving animals were approved by the ethics committee of the Central South University.

Oral glucose tolerance test

At the 6th weekend after dietary treatment, the rats were deprived of diet for 12 h, then given glucose solution (5 g/kg) by intragastric gavage. Blood samples were drawn from tails to do the oral glucose tolerance test (OGTT) test. Serum glucose was measured at 0, 30, 60, 90 and 120 min by fast blood glucose meter (OneTouch-Ⅱ, Johnson, America).

Assay for weight gain and fat index

After the OGTT test, the rats were fasted for 12 h and euthanized with ether. Liver, retroperitoneal (RET) and

epididymal (EPI) white adipose tissues were immediately removed and weighed.

Measurement: (1) Lee's index: (bodyweight)$^{1/3}$ × 10^3/ stem length (length from nasal tip to anus); (2) boby fat index: viscera fat/body weight ratio (viscera fat includes RET and EPI white adipose tissues); and (3) liver index: liver/body weight ratio.

Assay for serum triglycerides, total cholesterol and insulin

Blood samples were collected immediately in sterile tubes by heart puncture. Serum was collected by centrifugation at 2000 × *g* for 15 min at 4℃. The serum samples were analyzed for triglycerides (TG), total cholesterol (TCH) and insulin according to protocols of triglycerides fluid monoreagent (GPO-PAP) and cholesterol oxidase peroxidase-amidopyrine (CHOD-PAP) analysis (CHOD-PAP) and radioimmunoassay (Dongou Bio-Tech Ltd, Wenzhou and 3v Bio-Tech Ltd, Weifang, China).

Assay for liver lipid

Liver tissues (100 mg) were pulverized in liquid nitrogen to prepare 10% tissue homogenate. These homogenates were extracted under 4℃ with chloroform: methanol (2:1) for 48 h, then centrifuged at 12 000 × *g*/min for 15 min at 4℃. The concentrations of TG and TCH in supernatant were determined according to the protocols.

Liver histopathology

Liver tissues were routinely fixed in paraffin-embedded sections, and stained with hematoxylin and eosin (HE). To detect lipid droplets, sections were stained with Sudan Ⅳ and counterstained with hematoxylin. Each histologic section was observed for 5 fields of high power field. The classification and degree of fatty deposition are as follows: mild fatty degeneration (+): fatty hepatocytes occupying 30%-50% of the hepatic parenchyma, moderate fatty degeneration (++): 50%-75%, and severe fatty degeneration (+++): > 75%.

Statistical analysis

All data were presented as the mean ± SD. Univariate analysis of variance test was applied to determine the statistical significance of the difference among the groups, using the General Linear Models procedure of SPSS15.0 (SPSS Inc., Chicago, IL, USA). Some rank/frequency data were analyzed by nonparametric test, with the significance level set at $P < 0.05$.

RESULTS

Caloric intake, BW increment and obesity indexes in high-fat diet treated rats administered with different strains of Bifidobacteria

No rat died throughout the study. Caloric intake in control group (standard chow) was significantly lower than that of model group (HFD) (9055.68 ± 1246.62 kj *vs* 14562.32 ± 541.55 kj, $P < 0.05$). However, among model group and the four *Bifidobacteria* groups which were fed on high-fat diets, caloric intake showed no significant difference ($P > 0.05$). These indicate that the different groups of HFD rats had a similar caloric consumption (Figure 1A). The weight of all groups was increased every week, especially the model group and group *B. M13-4*. At the end of the 3rd wk, the BW increment in group *B. L66-5* was significantly less than in model group (90.26 ± 27.06 g *vs* 115.75 ± 15.13 g, $P < 0.05$), and further increased (156.05 ± 33.19 g *vs* 186.98 ± 16.25 g, $P < 0.05$) at the end of 5th wk. At the 6th week-end, weight increment in group *B. L66-5* was much less than the model group (175.19 ± 31.24 g *vs* 212.55 ± 18.54 g, $P < 0.05$). However, the BW increment was significantly higher in group *B. M13-4* than in the model group (264.27 ± 26.91 g *vs* 212.55 ± 18.54 g, $P < 0.05$). No significant change of BW was found in group *B. L75-4* and group *B. FS31-12* compared with the model group (Figure 1B).

Both the Lee's and liver indexes were decreased in all interventional groups. The liver index was obviously lower in control, *B. L75-4*, *B. M13-4* and *B. FS31-12* groups than in model group (4.04% ± 0.36%, 4.27% ± 0.50%, 4.27% ± 0.47%, 4.04% ± 0.36% *vs* 4.98% ± 0.72%, $P < 0.05$). The body lipid index showed no significant differences among the groups ($P > 0.05$) (Figure 1C). The Lee's index was significantly lower in groups *B. L66-5* and *B. FS31-12* than in model group (312.65 ± 20.18, 311.22 ± 17.52 *vs* 327.98 ± 8.90, $P < 0.05$, Figure 1D).

Effects of Bifidobacteria strains on glucose and lipid metabolism in high-fat diet treated rats

Effect of four *Bifidobacteria* strains on serum glucose: In control group, the peak of serum glucose appeared at 30 min and returned to normal at 120 min when glucose was significantly lower than in model group (5.32 ± 0.98 *vs* 7.17 ± 1.08, $P < 0.05$). In high-fat diet fed groups, the peak was postponed to 60-120 min and lasted longer than that in control group. In these groups, the serum glucose did not return to normal until 120 min. The serum glucose at 5 time points showed no significant differences among model group and interventional groups ($P > 0.05$) (Figure 2A).

Changes of serum insulin, TG and TCH and liver TG and TCH: The serum insulin (INS) showed no significant differences among the groups ($P > 0.05$) (Figure 2B). The TG levels in serum decreased significantly in all intervention groups compared with the model group (1.59 ± 0.73, 1.54 ± 0.30, 1.23 ± 0.65, 1.47 ± 0.70 *vs* 2.23 ± 0.76, $P < 0.05$), so did the TG levels in supernatant of liver homogenate in those groups (0.13 ± 0.02, 0.31 ± 0.16, 0.34 ± 0.08, 0.29 ± 0.10 *vs* 0.56 ± 0.04, $P < 0.05$) (Figure 2C). In groups *B. L66-5* and *B. FS31-12*, the TCH level in serum and liver were obviously lower than in model group (1.11 ± 0.18, 1.37 ± 0.26 *vs* 1.72 ± 0.38, $P < 0.05$ and 1.27 ± 0.08, 0.62 ± 0.6 *vs* 1.47 ± 0.05, $P < 0.05$, Figure 2D), while the other strains also showed a downward trend ($P > 0.05$).

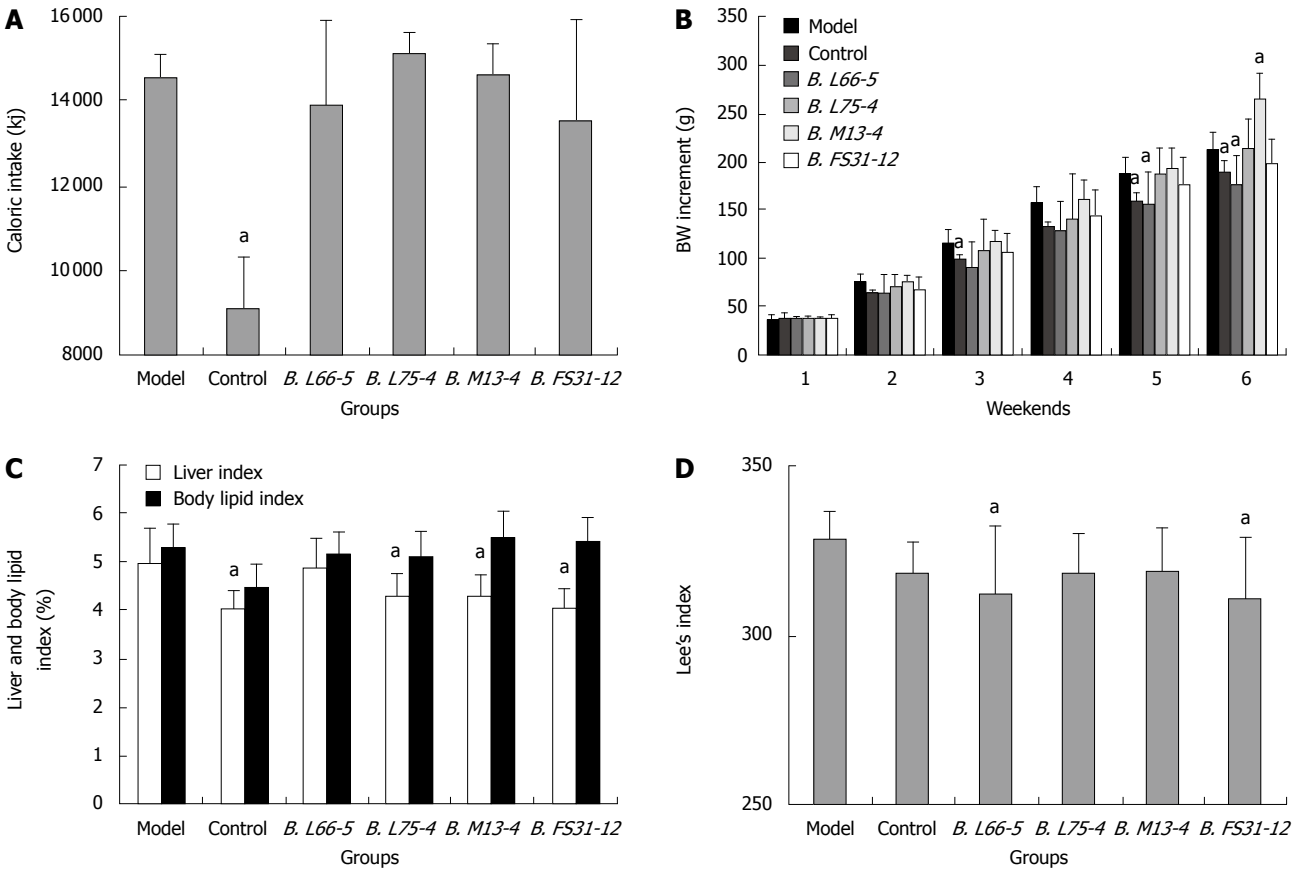

Figure 1 Caloric intake, body weight increment and obesity indexes in high-fat diet treated rats with different strains of *Bifidobacteria*. A: Sum of caloric intake in all groups at the 6th weekend; B: Body weight (BW) increment in all groups for 6 wk; C: Liver index and body lipid index in all groups; D: Lee's index in all groups. Results are shown as mean ± SD (n = 8). $^{a}P < 0.05$ *vs* model group.

Hepatic lipid deposition in high-fat diet induced rats treated with different strains of Bifidobacteria

Moderate degree of microvesicular steatosis was observed in model group. No fatty vacuolization was found in groups *B. L66-5* and *B. FS31-12*. Hepatocyte steatosis was obviously alleviated in groups *B. L75-4* and *B. M13-4* compared with model group (χ^2 = 30.754, P = 0.000) (Table 1 and Figure 3). Sudan Ⅳ staining showed that a plenty of scarlet lipid droplets deposited in the livers of model group, which confirmed the results shown in HE staining. Expectedly, lipid droplets were obviously decreased in all intervention groups compared with the model group, especially in groups *B. L66-5* and *B. FS31-12* (Figure 4).

DISCUSSION

To demonstrate the relationship between the administration of different strains of *Bifidobacteria* and the status of glucose and lipid metabolism, we established a murine obese model based on a 6-wk administration of high-fat diet (HFD), characterized by a significant increase of BW gain, fat mass, TG and TCH in serum and liver, and obesity indexes. Our results demonstrated that administration of the four *Bifidobacteria* (*Bifidobacteria L66-5*, *L75-4*, *M13-4* and *FS3-1-1-2*) played a role in reducing serum and liver TG and TCH, as well as liver lipid deposition. Furthermore, to our surprise, among the four strains of *Bifidobacteria*, two contrary results were yielded in BW changes: *B. M13-4* showed a significant increase in BW while *B. L66-5* showed a decrease in BW based on a similar caloric consumption. *Bifidobacteria L75-4* and *FS3-1-1-2* strains had no significant effect in BW change. However, all the four strains showed no significant influence on serum INS and glucose level. Based on our results, different *Bifidobacteria* strains lead to different responses of energy and fat metabolism in rat models.

Recent researches illustrated that gut microbiome should be considered as a set of genetic factors that, together with host genotype and life style (energy intake and expenditure), contribute to the pathophysiology of obesity. Turnbaugh *et al*[13] observed microbiota samples of obese mice, after transferring the microbiota to germ-free lean mice, significant fat gain was obtained and calorie extraction improved. BW increase has also been observed after administration of *Bifidobacterium*. In a preterm infant study in 1997, the authors added *Bifidobacterium breve* (about 0.5 × 10^9 live bacteria) to very low-birth weight infant formula, and their BW gain became significantly greater than in control group after administration for 4 wk[14]. In other cultures, *Lactobacillus rhamnosus GG* (1 × 10^7 cfu/g) also led to a weight growth in term infants after being supplemented to formulas for 4 mo[15]. However, *Bifidobacterium longum*[16], *Bifidobacterium lactis*[17,18], and *Bifidobacteria* combined with several species of *lactobacilli*

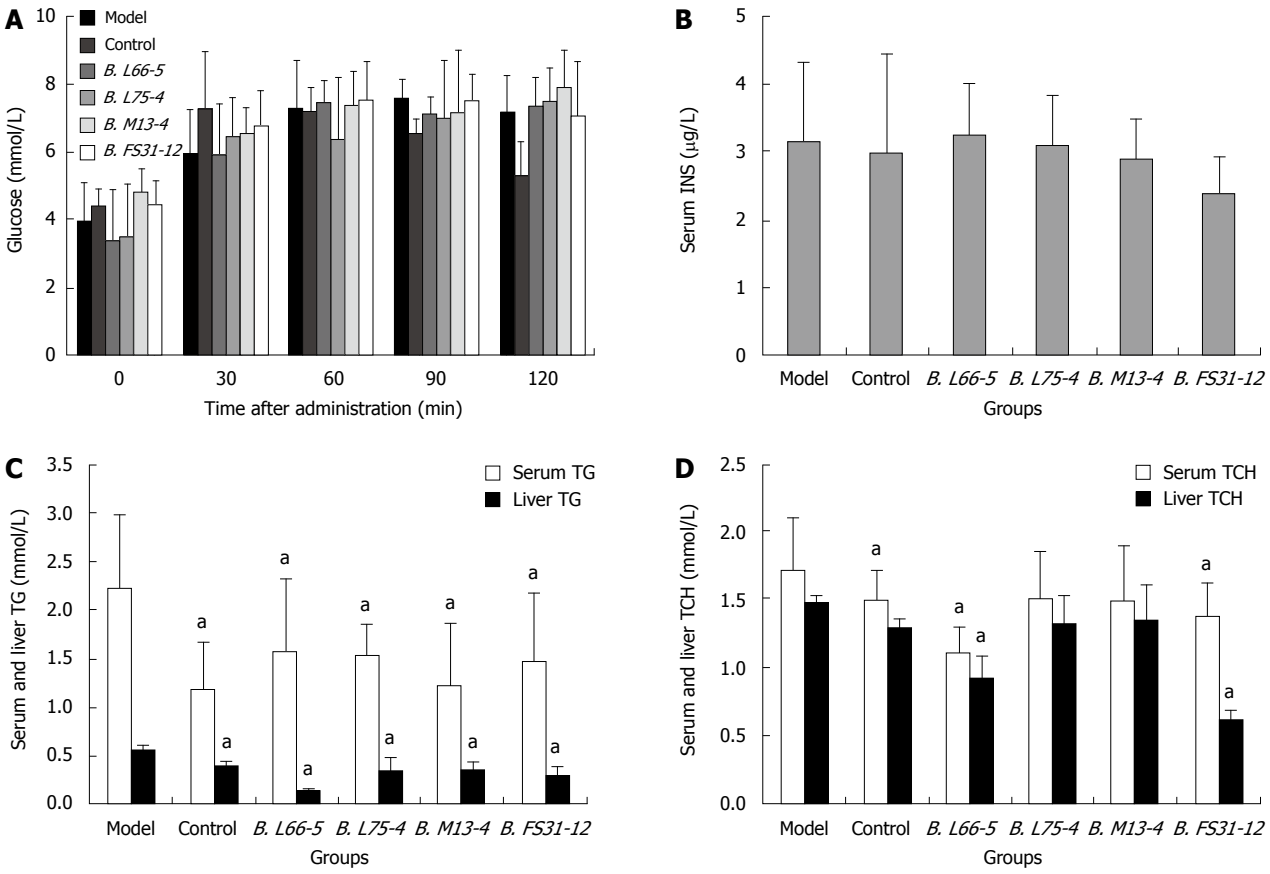

Figure 2 Effects of *Bifidobacteria* strains on glucose and lipid metabolism in high-fat diet treated rats. A: Effect of four *Bifidobacteria* strains on serum glucose; B: Serum insulin (INS) concentration in all groups; C: Serum and liver triglycerides (TG) in all groups; D: Serum and liver total cholesterol (TCH) in all groups. Results are shown as mean ± SD (n = 8), $^aP < 0.05$ *vs* model group.

Table 1 Degrees of liver lipid deposition in all groups

Groups	*n*	Degree of fatty deposition[1]			
		-	+	++	+++
Model	8	0	7	1	0
Control	8	8	0	0	0
B. L66-5	8	8	0	0	0
B. L75-4	8	6	2	0	0
B. M13-4	8	5	3	0	0
B. FS31-12	8	8	0	0	0

[1]The standard of classification and score for the degree of lipid deposition in liver was referred to the liver histopathology mentioned in MATERIALS AND METHODS.

plus *fructooligosaccharides*[19] have not shown any effects. Their similar concentrations and intervention periods indicated different effects *in vivo*. In this study, *B. M13-4* strain can decrease serum and liver TG, TCH and liver index, while no apparent changes were found in body lipid index and Lee's index with an obvious BW gain. It suggests that *B. M13-4* alleviated lipid deposition in liver although more fat was accumulated in the body. The mechanisms may be complex: (1) Intestinal microbiota can help the host to digest polysaccharides and absorb monosaccharides and short-chain fatty acids, which finally converse to lipids[20]; (2) Gut microbiota can modulate some signal pathways associated with energy balance in the gut epithelium: Gpr41, a short-chain fatty-acid binding G protein-coupled receptor, and peptide tyrosine tyrosine[21]; (3) *Bifidobacteria* can also help the host to eradicate *Campylobacter*[22] or *Eandida* and *Enterococcus*[23] to stabilize their intestinal flora; and (4) The amount of visceral fat is positively correlated with the insulin sensitivity[24,25]; the possible effect in improving insulin sensitivity to alleviating visceral adiposity of probiotics is limited in our study and worth further studies. Host colonized by *B. M13-4* absorbed more fat and transmitted them into body fat. This may contribute to the patients with fat/energy malabsorption. We should also reappraise the probiotics use in healthy and obese people for their potential effects such as fat/energy over-absorptions and weight over-growth.

To our surprise, rats colonized by *B. L66-5* showed a weight loss. The opposite outcomes in *B. M13-4* and *B. L66-5*, and strains showing no effect in BW, including *B. L75-4* and *B. FS3-1-1-2*, and other strains, including *Lactobacillus acidophilus ATCC 43121*[4], *Lactobacillus gasseri SBT2055*[5] and *Bifidobacterium longum*[6], *VSL#3*[12], *Lactobacillus reuteri*[26], may result from different interactions between strains and intestinal microbia, inappropriate dosage, variability in end-points, and subjects. Anyhow, *B. L66-5*

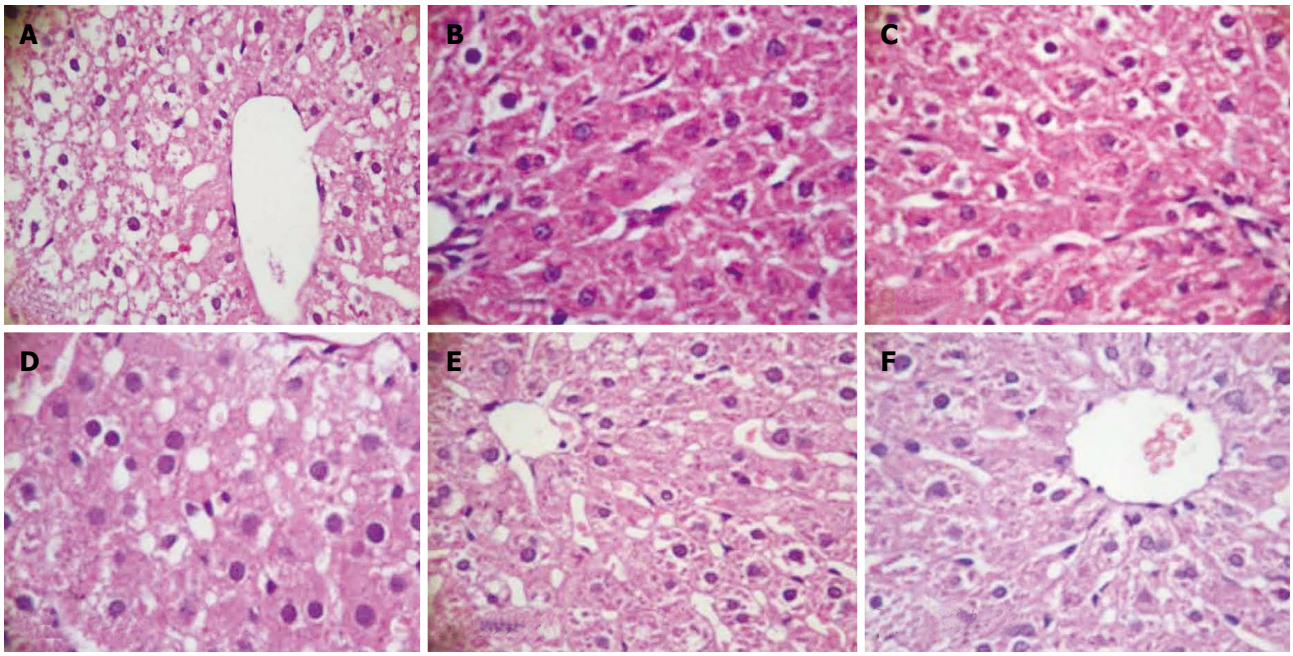

Figure 3 Hepatic tissue sections of each group in HE staining (HE, light microscope, × 400). A: Model; B: Control; C: *B. L66-5*; D: *B. L75-4*; E: *B. M13-4*; F: *B. FS31-12*.

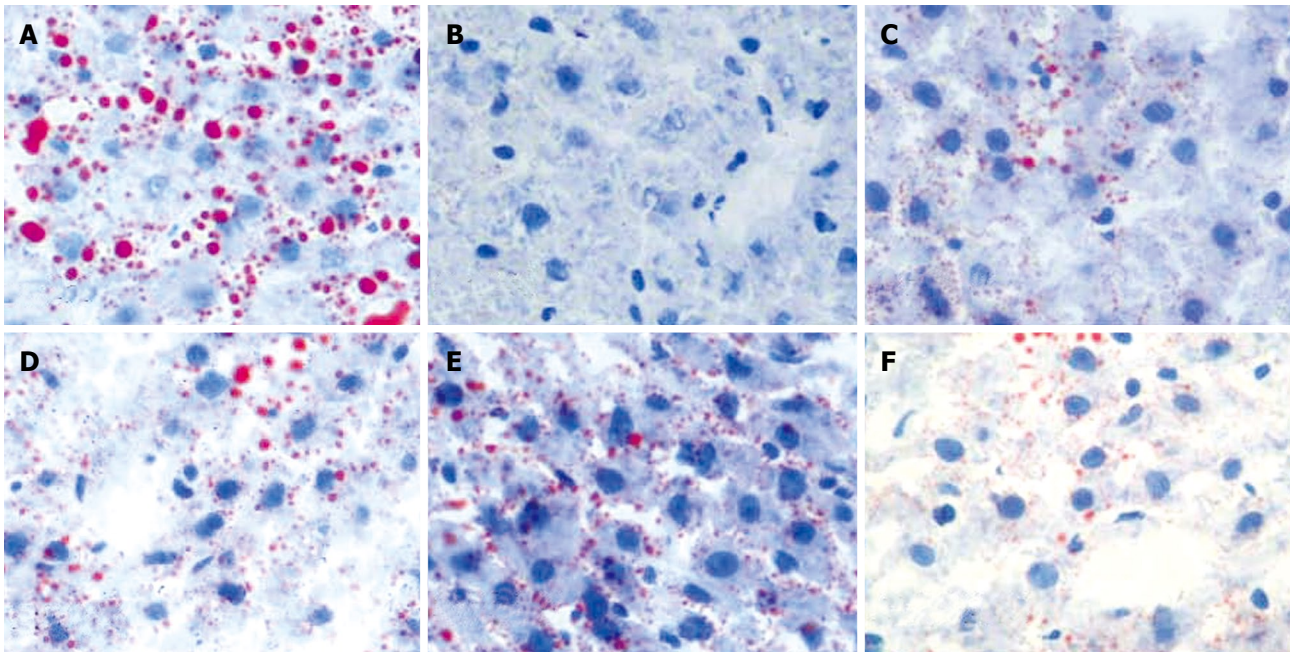

Figure 4 Hepatic tissue sections of each group in Sudan Ⅳ-staining (Sudan Ⅳ, light microscope, × 400), and lipid droplets are scarlet. A: Model; B: Control; C: *B. L66-5*; D: *B. L75-4*; E: *B. M13-4*; F: *B. FS31-12*.

was specific in regulating fat harvest and utilization, and may be a new therapeutic candidate for weight control.

All the four strains showed their effects in reducing serum TG and TCH, especially the strain *B. L66-5*. Administration of *B. L66-5* decreased the serum and liver TG and TCH and BW, and alleviated liver lipid deposition. Recent studies showed that probiotics had hypocholesteremia effects in both rat and human, including *Bifidobacterium longum*[6], *Lactobacillus acidophilus ATCC* 43121[4], *Lactobacillus plantarum MA2*[9], *Lactobacillus gasseri*[27], *Lactobacillus reuteri*[26,28], *Bacillus polyfermenticus SCD*[29], *etc*. Some specific probiotics strains could reduce serum TCH and TG, and increase the ratio of high-density lipoprotein/low-density lipoprotein (HDL/LDL). The mechanisms involved may be as follows: (1) assimilation of cholesterol by bacterial cells; (2) deconjugation of bile acids by bacterial acid hydrolyses (reduces cholesterol reabsorption, increases cholesterol excretion of deconjugated bile salts, and increases cholesterol uptake by low-density lipoprotein receptor pathway in the liver

as a compensatory response); (3) cholesterol binding to bacterial cell walls; and (4) inhibition of hepatic cholesterol synthesis and/or redistribution of cholesterol from plasma to the liver through the action of short-chain fatty acids, the end products of carbohydrate fermentation in the gut[30]. Furthermore, our results showed an individual amelioration effect in hepatic steatosis. The degree of fatty deposition in liver was obviously alleviated in all intervention groups as shown by liver histopathology. Probiotics VSL#3 also showed an effect in improving high-fat-diet induced hepatic steatosis in rats through lowering liver inflammatory signaling, increasing the expression of peroxisome proliferators-activated receptor α and hepatic natural killer T cell numbers[31,32]. However, the signaling capabilities of the four *Bifidobacteria* need further studies.

The four strains showed no significant differences in serum INS and glucose level. Similar negative results were also documented by Esposito *et al*[32] and Sato *et al*[5]. In our study, we fed the rats with high-fat diet for 6 wk, and none had a significant change of INS level, while in other HFD induced SD rats, insulin resistance did not occur until 8 mo or 36 wk[33,34]. So the next experimental period may prolong to 8 mo or longer.

In conclusion, we established a murine obese model based on a 6-wk administration of high-fat diet, which partially resembles the disorder of energy metabolism in human. The response of glucose and lipid metabolism to several strains of *Bifidobacteria* was evaluated. It was indicated that administration of the four strains of *Bifidobacteria* resulted in decreased serum/liver TG, serum/liver TCH, and hepatic steatosis, with no significant response to glucose and INS level. To our surprise, the data we presented demonstrated that administration of strain *B. L66-5* led to BW loss, decreased serum TG/TCH and decreased hepatic adiposity, while administration of strain *B. M13-4* resulted in significant increase of BW gain with alleviated hepatic adipose and serum/liver TG in rats. Thus, it is concluded that the response of energy metabolism to administration of *Bifidobacteria* is strain dependent. Different strains of *Bifidobacteria* might drive different directions of fat distribution. *B. M13-4* action may generate a new conception: certain probiotics may promote BW gain by more effective fat absorption, and a cautious assessment is needed before probiotics therapy is given, especially in obese people. *B. L66-5* might act as a new therapeutic probiotic candidate in controlling BW gain. Further studies should focus on evaluating how the administration of these *Bifidobacteria* modifies gut microbiota of obese rats.

COMMENTS

Background

Obesity is becoming a global epidemic. Recent researches have demonstrated that obesity may lead to the composition shift of gut microbiota in both mice and humans. The intentional manipulation of community structure of gut microbiota may be a novel strategy to treat obesity.

Research frontiers

Bifidobacterium is one of the most numerous "probiotic" in mammalian gut among commensal bacteria, and exhibited a significant effect in lowering serum total cholesterol. Specific strains of *Bifidobacteria* for energy metabolism may be helpful in management of obesity.

Innovations and breakthroughs

This study evaluates the effects of the administration of four strains of *Bifibofacteria* in obese rats. It demonstrated an interesting action of these strains on harvest energy from nutrients and regulation of lipid storage.

Applications

The manuscript gives new and interesting information about the key role of gut microbiota in the harvest energy from nutrients and regulation of lipid storage and metabolism.

Terminology

Probiotic bacteria are defined as living microorganisms that have beneficial effects in human health.

Peer review

This study evaluates the effects of the administration of four strains of *Bifibofacteria* on obese rats. It demonstrated an interesting action of these strains on harvest energy from nutrients and regulation of lipid storage. It is an interesting work that gives new information about the role of gut microbiota in host metabolism.

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BRIEF ARTICLE

Sessile serrated adenomas: Demographic, endoscopic and pathological characteristics

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Abstract

AIM: To study the demographic and endoscopic characteristics of patients with sessile serrated adenoma (SSA) in a single center.

METHODS: Patients with SSA were identified by review of the pathology database of Mayo Clinic Arizona from 2005 to 2007. A retrospective chart review was performed to extract data on demographics, polyp characteristics, presence of synchronous adenomatous polyps or cancer, polypectomy methods, and related complications.

RESULTS: One hundred and seventy-one (2.9%) of all patients undergoing colonoscopy had a total of 226 SSAs. The mean (SE) size of the SSAs was 8.1 (0.4) mm; 42% of SSAs were ≤ 5 mm, and 69% were ≤ 9 mm. Fifty-one per cent of SSAs were located in the cecum or ascending colon. Approximately half of the patients had synchronous polyps of other histological types, including hyperplastic and adenomatous polyps. Synchronous adenocarcinoma was present in seven (4%) cases. Ninety-seven percent of polyps were removed by colonoscopy.

CONCLUSION: Among patients with colon polyps, 2.9% were found to have SSAs. Most of the SSAs were located in the right side and were safely managed by colonoscopy.

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Key words: Sessile serrated polyp; Sessile serrated adenoma; Colonoscopy

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INTRODUCTION

Colorectal carcinoma (CRC) follows the adenoma-carcinoma sequence[1] in the majority of patients. CRC development secondary to DNA microsatellite instability (MSI-high) with a deficiency of DNA mismatch repair occurs only in a minority of patients[2,3]. MSI-high carcinoma usually progresses along the "serrated pathway"[4-6]. Historically, hyperplastic polyps (HPs) have usually been regarded as non-neoplastic lesions with no malignant

potential. However, some lesions previously diagnosed as HP but with malignant potential, which are now known as "sessile serrated adenomas" (SSAs), are increasingly being recognized[7,8]. SSAs may develop MSI as they progress toward carcinoma, and are potential precursors of sporadic microsatellite unstable CRC. Clinical and demographic characteristics including age, sex, racial distribution, endoscopic characteristics (location, gross appearance), recurrence rate, and the rate of progression of these polyps to carcinoma are not well known. There are no published consensus practice guidelines, and the optimal surveillance colonoscopy interval of these polyps is unknown, due to the paucity of clinical information regarding these patients.

The aim of our study was to identify demographic and endoscopic characteristics of patients with SSA in a single center and report the clinical experience with SSA in a large group practice.

MATERIALS AND METHODS

Patients with SSA were identified by review of an institutional pathology database from the years 2005-2007. Patient charts were reviewed for data on demographics and colonoscopy details. Colonoscopy reports were reviewed to obtain the number, size, and location of polyps, as well as the morphological appearance and the polypectomy methods. In addition, data were collected on synchronous adenomatous polyps or cancer, and the number of colonoscopies needed to eradicate the polyps, the need for surgical therapy, and colonoscopy-related complications. All colonoscopies were done with standard white light examination technique with standard definition colonoscopes. SSA was defined in practice and in this report as polypoid lesions that lacked typical or conventional dysplasia, but that showed architectural features of disordered growth[9,10]. These features are: basal crypt dilatation, horizontal orientation of deep crypts, prominent serration extending deep into the crypts, irregular crypt branching, and inverted crypts. Other criteria consistent with abnormal proliferation were also useful in the diagnosis of SSA; these were nuclear atypia and/or oval nuclei in mid/upper crypts, prominent nucleoli in middle/superficial crypts, dystrophic goblet cells, irregular distribution of goblet cells, mitoses in mid/upper crypts, and excessive crypt or luminal mucin (Figure 1). We classified the polyps as SSA even if the diagnostic changes were only identified focally.

Descriptive analysis was performed using statistical software (SPSS version 17; SPSS Inc., Chicago, IL, USA) to study the demographic and clinical characteristics of these patients.

RESULTS

Among patients undergoing colonoscopy from 2005 to 2007 (21 238 colonoscopies), a total of 5991 patients were found to have polyps. Of these, 171 (2.9%) patients had a total of 226 SSAs. The majority of patients with SSAs in our study were Caucasian (164, 96%) with a mean (SE) age of 65.9 (0.8) years and a mean body mass index of 29.6 (0.4). Ninety-one patients (53%) were male. The SSA characteristics are summarized in Table 1. The mean size of the SSAs was 8.1 (0.4) mm (range 2-40 mm); 42% were ⩽ 5 mm and 69% were ⩽ 9 mm in size. The majority of SSAs were located in the right side of the colon (Figure 2). All SSAs were described as sessile or flat in appearance (Figure 3). The mean number of SSAs per patient was three (range 1-24); 58 (34%) patients had one SSA, 43 (25%) had two and 70 (41%) had three or more. Approximately half (87, 51%) of the patients had synchronous polyps of other histological appearance. The most common type of associated pathology in these synchronous polyps was tubular adenoma in 62 (49%) polyps, tubulovillous adenoma (TVA) in 13 (10%) and hyperplastic pathology in 50 (40%). One polyp was a small carcinoid. Synchronous adenocarcinoma was present in seven (4%) cases and all of these cancers were present in the cecum or ascending colon.

Thirty-one percent of SSAs were removed by cold biopsy/cold snare, 4% by hot biopsy, 63% by snare cautery, and 2.7% required surgical excision due to size or associated malignancy. Polyp removal techniques depended on the judgment and preference of the endoscopist. All polyps ⩾ 2 cm in size were removed by saline-assisted polypectomy. There were no complications associated with the endoscopic resection of the SSAs. Although up to nine pathologists interpreted the histopathology of these polyps, the majority (75%) were diagnosed as SSA by two

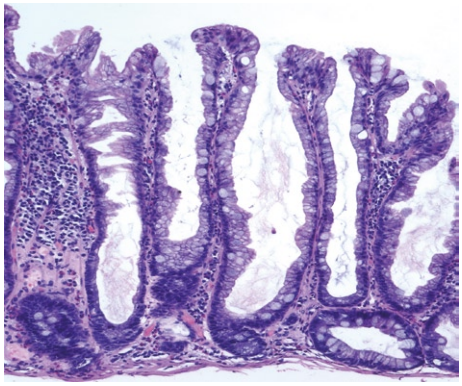

Figure 1 Sessile serrated adenoma. Flask-shaped glands with dilated and irregular architecture of the gland bases with abundant luminal mucin.

Table 1 Sessile serrated adenoma characteristics

Mean size of SSA	8.1 mm (range 2-40 mm) ⩽ 5 mm (42%), ⩽ 9 mm (69%)
Mean No. of SSAs per patient	3 (range 1-24)
Location of SSAs	51%: Cecum, ascending colon 49%: Remaining colon
Appearance	Flat or sessile (all polyps)
Synchronous polyps of other histology	Present in 87 (51%) patients TA: 62 (49%) TVA: 13 (10%) HP: 50 (40%)
Synchronous adenocarcinoma	7 (4%), all in right side of the colon

SSA: Sessile serrated adenoma; TA: Tubular adenoma; TVA: Tubulo-villous adenoma; HP: Hyperplastic polyp.

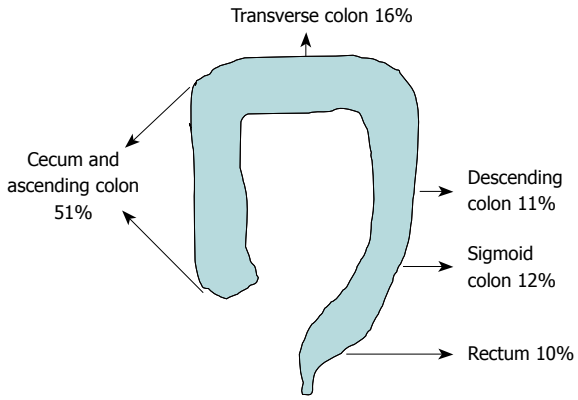

Figure 2 Distribution of sessile serrated adenomas in patient population.

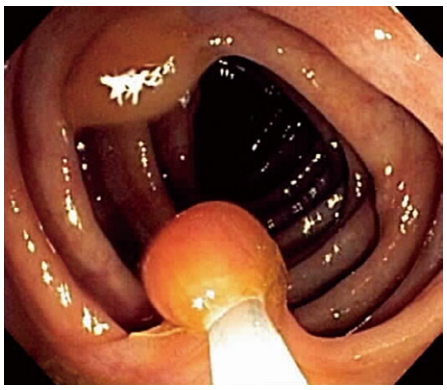

Figure 3 Colonoscopic image of sessile serrated adenoma (sessile, flat with yellow appearance).

highly experienced gastrointestinal pathologists. In the clinical practice of pathologists interpreting specimens, in 12.6% cases, the diagnosis of SSA was confirmed in consultation by a second pathologist within the department.

DISCUSSION

Colorectal serrated polyps are a group of morphologically related lesions that include aberrant crypt foci, conventional HPs, advanced serrated adenomas (ASAs)[11] and SSAs. These lesions share some histological features, however, they differ significantly at the molecular level, and are biologically distinct[12]. In 1990, Longacre and Fenoglio-Preiser[13] first used the term serrated adenoma. While many patients probably had serrated lesions described as hyperplastic in the past, some have mixed hyperplastic and adenomatous features. Unfortunately, often there is significant disagreement among pathologists in the diagnosis of these polyps. In a study of 185 serrated polyps, five gastrointestinal pathologists had only moderate overall agreement ($\kappa = 0.58$), with near perfect agreement on traditional serrated adenoma, also called ASA, but varying most in the SSA and hyperplastic categories[14]. In our study, the majority of these polyps (75%) were diagnosed as SSA by two experienced gastrointestinal pathologists, however, up to nine pathologists interpreted the histopathology of polyps. In 12.6% of our cases, a second pathologist's opinion was obtained by intradepartmental consultation for diagnosing SSA, which suggests difficulty in day to day clinical interpretation using standard published criteria. Simple standardized diagnostic criteria and terminology could improve interobserver agreement among pathologists, and represent a limitation in the interpretation of this study, and an opportunity for improvement in overall clinical practice.

In the present study, approximately 3% of all polyps were SSAs, which is similar to previous studies[15,16]. However, a prospective study using magnifying chromoendoscopy has reported a 9% prevalence of SSAs[17]. All examinations in our study were performed with standard white light colonoscopes, and the diagnosis of SSAs was made in real time clinical practice between 2005 and 2007. Polyps in the database were not re-examined, which could have changed the actual prevalence. Endoscopically, SSAs commonly appear flat or sessile, have a soft, smooth surface, and are often covered with mucus, which gives an initial yellow appearance[9]. Small, flat, right-sided lesions potentially can be missed by white light colonoscopy. Newer techniques such as high definition colonoscopy, narrow band imaging or chromoendoscopy could help to distinguish these polyps from normal mucosa.

The majority of patients with SSA in our study had multiple SSAs (mean = 3, range = 1-24). Approximately half of these patients also had synchronous polyps of other histological type, such as tubular adenoma, TVA and HPs. Synchronous right-sided adenocarcinoma was seen in 4% of cases.

As noted in other studies[15,18,19], the majority (67%) of SSAs in our study were located proximal to the splenic flexure. Forty-two percent of all SSA were $\leqslant$ 5 mm, and 69% were $\leqslant$ 9 mm in size. In a study of 13 992 colonoscopies from the Clinical Outcomes Research Initiative (CORI) repository[20], when pathology results were available, 1.7% of polyps that measured 5 mm ($n = 3744$) had advanced neoplasia [one cancer, one high-grade dysplasia (HGD), 44 TVA]. In polyps 6-9 mm in size ($n = 1198$), 6.6% had advanced neoplasia (two cancers, nine HGD, and 53 TVA), which suggests that not all small polyps are innocent. The natural history of SSAs is not well understood. Although carcinoma associated with advanced serrated adenoma has been reported to be a distinct type of neoplasm, which accounts for 5.8%-7.5% of all colorectal carcinomas and up to 17.5% of proximal colon cancers[21,22], the overall impact of SSAs on cancer risk is not known because of a lack of data on their true prevalence. There have been reports of rapid progression of SSA to invasive cancer as early as within 8 mo [23]. The current surveillance interval recommendation[24,25] for small SSAs is mainly based on indirect evidence, and is similar to that for small adenoma (5 years). Surveillance of large SSAs or more than three SSAs is similar to large or advanced adenoma (3 years).

The retrospective design and lack of long-term longitudinal follow-up data are major limitations of our study. Demographically, our patient population was predominantly Caucasian and above ideal body weight. These factors could limit the applicability of our results to other populations. Future large prospective studies are needed to understand the natural history of SSAs and to establish

clinical practice guidelines for optimal cost-effective management and surveillance of patients with SSA.

COMMENTS

Background

Colorectal carcinoma (CRC) usually follows a sequence of development from adenomatous polyp to carcinoma. In a minority of patients, CRC development occurs due to alterations of small areas of DNA known as microsatellite instability (MSI). These patients have a defect in their DNA repair mechanism. Carcinomas that develop by this mechanism often have a special serrated appearance. One type of polyp with a serrated appearance, hyperplastic polyps (HPs), have usually been regarded as non-neoplastic lesions with no malignant potential. However, some lesions previously diagnosed as HPs, but with malignant potential, are now known as sessile serrated adenomas (SSAs), and are increasingly being recognized. SSAs can develop MSI as they progress toward carcinoma, and are potential precursors of sporadic microsatellite unstable CRC.

Research frontiers

SSA are uncommon colon polyps. The clinical and demographic characteristics of patients with these polyps, including age, sex, racial distribution, endoscopic characteristics (location, gross appearance), recurrence rate, and rate of progression of these polyps to carcinoma are not well known, and were evaluated in this study.

Innovations and breakthroughs

Between 2005 and 2007, 171 (2.9%) patients undergoing colonoscopy had a total of 226 SSAs. The average size of the SSAs was 8.1 mm. Forty-two percent of SSAs were ⩽ 5 mm and 69% were ⩽ 9 mm. Fifty-one per cent of SSAs were located in the cecum or ascending colon. Approximately half of the patients had coexisting colon polyps of other histological types, including hyperplastic and adenomatous polyps. Coexisting adenocarcinoma was present in seven (4%) cases. Ninety-seven percent of polyps were removed by colonoscopy.

Applications

Most of the SSAs were located in the right side of the colon and were safely managed by colonoscopy. Concurrent lesions including adenomas and right-sided colon cancers were not uncommon. The natural history of SSAs is being learned, and greater concern about small polyps might exist in the future, as some can be aggressive or associated with other lesions.

Peer review

This is an extensive study of a very important topic. The disadvantage of the study is its retrospective nature; especially concerning the endoscopic findings and follow-up.

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BRIEF ARTICLE

Can chronic gastritis cause an increase in fecal calprotectin concentrations?

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Abstract

AIM: To evaluate fecal calprotectin concentrations (FCCs) in subjects with chronic gastritis and the correlation between FCCs and gastritis activity score.

METHODS: FCCs were measured in 61 patients with histological diagnosis of gastritis and in 74 healthy volunteers. Histological grading of gastritis was performed according to the updated Sydney gastritis classification. Patients were subdivided into 2 groups according to the presence/absence of an active gastritis. Patients with chronic active gastritis were divided into 3 subgroups on the basis of the activity score (mild, moderate, marked). FFCs in relation to *Helicobacter pylori* (*H. pylori*) infection and proton pump inhibitor (PPI) use were also evaluated.

RESULTS: FCCs in patients with chronic active gastritis were not significantly different to FCCs either in subjects with non active gastritis or in healthy controls. Among patients with chronic active gastritis (even marked), FCCs did not significantly differ according to activity score. No significant differences in FCCs were found when considering *H. pylori*, as well as when considering PPI chronic use.

CONCLUSION: FCCs were not significantly increased in subjects with chronic gastritis, even in those patients with a marked neutrophil infiltration.

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Key words: Chronic gastritis; Fecal calprotectin; Intestinal inflammation; Neutrophils

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INTRODUCTION

Calprotectin is a calcium and zinc binding protein, mainly contained in neutrophils where it accounts for more than 60% of cytosolic proteins. It has well-known antimicrobial activity, both bacterial and fungicidal[1]. Elevated concentrations of calprotectin can be measured in plasma, synovial fluid, urine, liquor, saliva and feces when an

inflammation process with recruitment of neutrophils is ongoing[2,3]. In particular, the presence of calprotectin in feces quantitatively relates to neutrophil migration towards the gastrointestinal tract[4]. Its levels are closely correlated with the fecal excretion of ^{111}In-labelled leukocytes[5]. Therefore, it is considered a useful marker of intestinal inflammation[6]. Several recent studies reported a significant increase in fecal calprotectin concentrations (FCCs) in intestinal conditions characterized by a conspicuous neutrophil infiltrate, such as inflammatory bowel diseases (IBDs) and non-steroidal antiinflammatory drug (NSAID) enteropathy[7-9]. It may accurately distinguish IBD from non-IBD conditions (such as irritable bowel syndrome)[10,11]. It has also been proposed as a reliable marker able to predict clinical relapse in IBD patients[12,13]. Diagnostic accuracy of FCCs in colorectal neoplasia has not been univocally established yet[14].

Chronic gastritis represents a common and heterogeneous inflammatory process. It can be morphologically characterized by a variable inflammatory infiltrate in the lamina propria, within the epithelium and within the foveolar lumen[15]. According to the updated Sydney System, the presence of a neutrophil infiltrate characterizes the "activity" of gastritis[15].

The aim of our study was to evaluate FCCs in subjects with chronic gastritis and the possible correlation between FCCs and the activity score, according to the updated Sydney System gastritis classification.

MATERIALS AND METHODS

Patients

Between May 2008 and December 2008, subjects who were referred to the Endoscopy Center of "Gemelli Hospital" for upper gastrointestinal endoscopy, were invited to enter the study. In those subjects who agreed to participate in the study, the extraction of at least 5 biopsy samples (2 from the antrum, 2 from the corpus and one from the incisura angularis) had been undertaken to correctly characterize an eventual gastritis process, in accordance with Sydney's recommendations[15]. However, when esophageal lesions, gastric ulcers, gastric polyps or duodenal lesions were found during the endoscopy, the necessary biopsy specimens were taken, and these subjects were not included in the study. In addition, subjects with IBDs or family history of IBDs, colorectal cancer, chronic use of NSAIDs, history of gastric resection, coexisting and severe cardiopulmonary, hepatic, renal, neurologic, psychiatric, endocrine and rheumatologic diseases, malignancy, pregnancy, alcohol abuse, other intestinal disorders characterized by increased mucosal permeability and inflammatory changes, were not considered for the study.

All the eligible subjects were asked to provide a stool sample for measurement of calprotectin levels, within 2 d of endoscopic examination, before starting specific therapy. Stools were also examined to exclude infectious intestinal diseases. All subjects were asked if they were taking proton pump inhibitor (PPI) therapy for at least since 1 mo before the endoscopy.

According to the updated Sydney System, depending on the presence/absence of a neutrophil infiltrate, patients with chronic gastritis were divided into 2 groups: group A which consisted of patients with active gastritis and group B which consisted of patients with non active gastritis. Furthermore, adult healthy volunteers participated as a further control group, providing their own stool sample.

Procedures were in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983. Each subject gave written informed consent for the study. The study was approved by the Institutional Board of Department of Internal Medicine, Catholic University of Rome.

Histological evaluation

The biopsy samples were fixed in 4% buffered formalin, processed in the usual manner, and paraffin embedded. The sections were stained with hematoxylin and eosin for histological evaluation; Giemsa stain was also used to evaluate the presence of *Helicobacter pylori* (*H. pylori*). The sections were evaluated by 2 separate expert gastrointestinal pathologists working blind. The degree of activity of inflammation was assessed using a semiquantitative 3-tiered scale (mild, moderate, marked) according to the updated Sydney System[15]. The infiltration of neutrophil granulocytes was defined as "mild" when isolated cells of this type were identified in the lamina propria only with difficulty, after a thorough search; it was defined as "moderate" if neutrophils were either easily detectable in the lamina propria or were found within the epithelium, provided they were not crowded; finally, the infiltration was defined as "marked" when a dense neutrophil infiltrate, usually involving both lamina propria and epithelium, was strikingly evident at low power magnification. When activity differed among antrum, corpus and incisura angularis, the activity grade in the most severely affected compartment was considered; when activity grade changed among different biopsies of the same gastric compartment, the predominant grade was considered, according to the updated Sydney classification[16]. *H. pylori* status was evaluated as present/absent in all the examined biopsy samples. *H. pylori* density score was graded as mild, moderate and marked, according to the updated Sydney classification[16].

Fecal calprotectin measurement

Each subject was instructed to collect and return a single stool sample within 48 h of defecation. Upon receipt, the stools were frozen and stored at -20℃ for subsequent biomarker determination.

The stool samples were prepared and analyzed according to the manufacturer's instructions (Calprest; Eurospital SpA, Trieste, Italy). A portion of each sample (40-120 mg) was measured and an extraction buffer containing citrate and urea was added in a weight per volume ratio of 1:50. The samples were mixed for 30 s by a vortex method and homogenized for 25 min. One

Table 1 Demographic data and mean fecal calprotectin concentrations of the different study groups (mean ± SD)

Groups	*n*	Sex (M/F)	Age (yr)	FCCs (μg/g)
Patients	61	28/33	49.64 ± 13.80	28.25 ± 23.43
Active gastritis	35	15/20	49.66 ± 14.15	29.70 ± 21.26
Mild	15	6/9	48.07 ± 14.56	31.44 ± 22.55
Moderate	10	7/3	47.60 ± 13.72	31.08 ± 23.68
Marked	10	2/8	53.30 ± 14.64	26.57 ± 17.66
Non active gastritis	26	13/13	49.90 ± 13.61	25.97 ± 22.55
Healthy controls	74	32/42	45.93 ± 12.42	31.20 ± 19.18

FCCs: Fecal calprotectin concentrations.

milliliter of the homogenate was transferred to a tube and centrifuged for 20 min. Finally, the supernatant was collected and frozen at -20℃. In most cases, time from sampling to preparation and freezing was estimated to be 1-3 d, except for a few samples that took 4-6 d before handling. The supernatants were thawed and analyzed later with Calprest, a quantitative calprotectin ELISA, for determination of calprotectin in stools. The within-assay coefficient of variation was 1.5%. Calprotectin was expressed as μg/g of feces.

Statistical analysis

Statistical comparison of age and sex among patients with chronic active gastritis, non active gastritis and healthy controls was performed by the *t*-test for unpaired data and χ^2 test. FCCs among subjects with active gastritis, non active gastritis and healthy controls groups were compared by the *t*-test for unpaired data.

In subjects with chronic active gastritis, FCC differences among the subgroups identified by density of neutrophil infiltration (activity score) were analyzed by means of one-way analysis of variance (ANOVA). The *post hoc* effect was assessed by Bonferroni *t*-test. Comparison between FCCs and *H. pylori* status, FCCs and PPI use, was performed by means of the *t*-test for unpaired data. FCC differences among the subgroups identified by density of *H. pylori* infection were analyzed by ANOVA. The *post hoc* effect was assessed by the Bonferroni *t*-test. The statistical analysis of categorical parameters was performed by the χ^2 test. All values were assessed as mean ± SD. A *P*-value of 0.05 or less was regarded as significant.

RESULTS

During the study period, 929 subjects had an upper intestinal endoscopy; 696 were ruled out on the basis of the above-mentioned exclusion criteria. Of the 247 eligible patients, 61 Caucasians (28 male, 33 female, mean age 49.64 ± 13.80 years) gave their consent to participate in the study. Seventy four adult healthy volunteers (32 male, 42 female, mean age 45.93 ± 12.42 years) entered the study as controls. The demographic data of the different study groups are summarized in Table 1. There were no significant differences between the groups regarding age and sex.

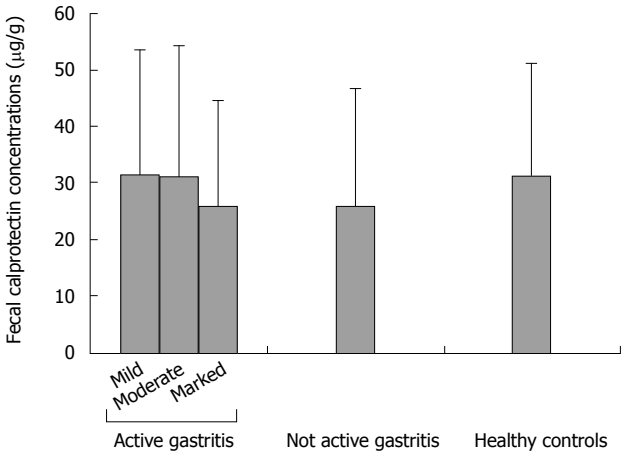

Figure 1 Mean fecal calprotectin concentrations ± SD in the different study groups.

According to the updated Sydney System classification, 35 patients showed chronic active gastritis (group A); in particular, 15 showed mild activity, 10 moderate activity and 10 marked activity. When separately analyzed by antrum gastritis activity (AGA) and corpus gastritis activity (CGA), 21 patients showed predominant antral activity (4 mild AGA without CGA; 7 moderate AGA, 6 with mild CGA and 1 without CGA; 10 marked AGA, 7 with mild CGA and 3 with moderate CGA), 2 showed predominant corpus activity (both with mild CGA without AGA), and the other 12 patients showed a concordant activity between antrum and corpus (9 mild and 3 moderate in both AGA and CGA).

Of the 26 patients with non active chronic gastritis (group B), 15 showed a predominant chronic mononuclear infiltrate, 4 intestinal metaplasia and 7 glandular atrophy.

Mean FCCs were not significantly different between group A and group B, and they both did not differ significantly from FCCs in healthy volunteers (*P* = NS for all comparisons).

When considering only patients with chronic active gastritis, mean FCCs were not significantly different among the 3 subgroups identified by the different degree of neutrophil infiltrate (Table 1 and Figure 1). Also, when separately considering antrum and corpus gastritis, mean FCCs did not correlate with the degree of activity in either subgroup.

When considering the presence of *H. pylori* infection in the whole study group, 24 patients were *H. pylori* positive (7 with mild infection, 8 moderate and 9 marked), while 37 patients were *H. pylori* negative; mean FCCs neither significantly differed between the 2 subgroups (27.35 ± 22.64 *vs* 28.84 ± 24.21, *P* = NS), nor correlated with degree of *H. pylori* infection (*P* = NS for all comparisons). On the other hand, both the presence and density of *H. pylori* significantly correlated with neutrophilic infiltration. In particular, in subjects with chronic active gastritis, 5/15 (33%) with a mild active gastritis, 8/10 (80%) with a moderate active gastritis, and 10/10 (100%) with a severe active gastritis, were *H. pylori* positive, whereas in the group

with non active gastritis, only 1/26 (3.8%) was *H. pylori* positive ($P < 0.001$). In addition, when considering *H. pylori* density, of the 7 patients with a mild *H. pylori* density score, 4 showed mild active gastritis and 3 moderate active gastritis; of the 8 patients with moderate *H. pylori* density, 3 showed moderate active gastritis and 4 showed marked active gastritis, while one had non active gastritis; of the 9 patients with marked *H. pylori* density, one showed mild active gastritis, 2 moderate active gastritis and 6 marked active gastritis ($P < 0.05$).

Finally, when considering PPI use, 22 patients were on PPI therapy and 39 patients were not; mean FCCs were not significantly different between the 2 groups (32.88 ± 25.90 *vs* 25.64 ± 25.83, P = NS).

DISCUSSION

Our study showed no significant differences in FCCs between patients with chronic active gastritis and non active chronic gastritis controls, regardless of the degree of neutrophil infiltration. In addition, FCCs in both groups did not significantly differ with regard to that in healthy controls.

Fecal calprotectin has recently emerged as a reliable marker of intestinal inflammation[14]. Different studies regarding fecal calprotectin have been carried out in bowel diseases, mainly IBDs[8-11]. Up to now, no specific studies have been designed to evaluate FCCs in upper gastrointestinal tract diseases. The few available data on this topic can only be gathered from studies evaluating FCCs in different conditions throughout the gastrointestinal tract. In this regard, only Summerton *et al*[17], in 2002, performed a study evaluating FCCs in different gastrointestinal inflammatory and cancer conditions. In particular, 26 patients showed upper gastrointestinal inflammation because of gastritis and duodenitis. FCCs were in the normal range in all these subjects. Nevertheless, a correlation between FCCs and histological severity of inflammation was not performed in this study.

Chronic gastritis is a very common clinical condition. The updated Sydney System provided the term "activity" as an expression of the presence of neutrophils on a background of chronic inflammation[15].

As expected, we found that patients with non active chronic gastritis did not show increased FCCs, since a neutrophil infiltrate is lacking in these conditions. Nevertheless, we also found that FCCs were not significantly increased in active chronic gastritis, even in subjects with a marked activity score (and so a high grade of neutrophil infiltration). This result could be explained by the consideration that the inflammatory process, and in particular the neutrophil recruitment occurring in gastritis, is far less severe than in other intestinal conditions, mainly IBDs. Furthermore, our findings can be also explained by the smaller extent of inflamed tissue found in gastritis with respect to that in IBDs. In this regard, Sipponenn *et al*[18] reported that subjects with ileal Crohn's disease showed lower fecal markers (calprotectin and lactoferrin) compared to subjects with colonic involvement. They supposed that this finding might be explained by the limited extent of ileal disease, even in the presence of endoscopic and histological inflammation. In addition, in 39 children with IBDs, it has been shown that FCCs were closely related not only to disease severity, but also to disease extent[19].

In our study, we did not consider all those subjects with endoscopic findings involving the esophagus, duodenum or with gastric polyps and ulcers, because our aim was to evaluate FCCs only in chronic gastritis, relating these levels to the neutrophil infiltrate classified according to a validated histological score. Further studies, specifically aimed at this purpose, should clarify if FCCs might be increased in other upper gastrointestinal diseases different from chronic gastritis.

It has been reported that a neutrophil infiltrate is almost always present in *H. pylori* gastritis and usually disappears within a few days of antibiotic therapy[20]. In agreement with data in the literature, we found that the gastritis activity score was closely correlated with the degree of *H. pylori* infection. In particular, only one patient with an absent neutrophil infiltrate was *H. pylori* positive, while all 10 patients with marked active gastritis showed *H. pylori* infection. However, no significant differences were found when FCCs was compared between *H. pylori*-positive and *H. pylori*-negative subjects, regardless of *H. pylori* density score.

Concerning the relationship between PPI therapy and FCCs, Poullis *et al*[21], in a letter, reported that patients using PPIs had significantly higher FFCs compared to those not on PPIs. Nevertheless, data on their population were lacking; they did not undergo endoscopy, and other causes of increased FCCs, such as IBDs and NSAID use were not excluded. On the other hand, it has been reported that PPIs may also inhibit proton pumps present on membranes of phagolysosomes of neutrophils, interfering with neutrophil release of reactive oxygen species, commonly mediated by lysosomal acidification[22,23]. We found that FCCs were not significantly different between subjects taking PPIs and subjects who did not, thus suggesting that gastric pH is unlikely to be responsible for the low levels of FCC we found. On the other hand, it is not possible to exclude that gastric acidity could interfere with FCCs. Until now, no data have been available on this topic and further studies should be encouraged.

In conclusion, we showed that in subjects with chronic active gastritis, even marked, FCCs were not significantly increased when compared with FCCs either in subjects with non active gastritis or in healthy controls. Thus we recommend that in subjects with high FCCs, causes of gut inflammation other than chronic gastritis should be checked.

COMMENTS

Background

Fecal calprotectin is a valid marker of intestinal inflammation, being quantitatively related to neutrophil migration towards the gastrointestinal tract. Fecal calprotectin concentrations (FCCs) are significantly increased in intestinal diseases characterized by a conspicuous neutrophil infiltration, mainly inflam-

matory bowel diseases. Chronic gastritis morphologically shows a variable neutrophil infiltrate, which characterizes the gastritis activity, according to the updated Sydney System classification.

Research frontiers

No systematic study has ever been performed to evaluate FCCs in subjects with chronic gastritis and the possible correlation between FCCs and gastritis activity score.

Innovations and breakthroughs

The authors found no significant difference between FCCs in patients with chronic active gastritis and FCCs either in subjects with non active gastritis or in healthy controls. Among patients with chronic active gastritis (even marked), FCCs did not correlate with the activity score.

Applications

The authors recommend that in subject with high FCCs, causes of gut inflammation other than chronic gastritis should be checked.

Terminology

Calprotectin: A calcium and zinc binding protein, mainly contained in neutrophils where it accounts for more than 60% of cytosolic proteins. It has well-known antimicrobial activity, both bacterial and fungicidal. Elevated concentrations of calprotectin can be measured in plasma, synovial fluid, urine, liquor, saliva and feces when an inflammation process with recruitment of neutrophils is ongoing. The presence of calprotectin in feces quantitatively relates to neutrophil migration towards the gastrointestinal tract. Active gastritis: according to the updated Sydney System, the presence of a neutrophil infiltrate characterizes the "activity" of gastritis.

Peer review

The study is set up correctly. The material studied is big enough to allow conclusions to be drawn. The paper is written sufficiently well, the Introduction gives a good overview of the study background and the authors clearly raised the hypothesis of the study. The description of the method and material studied is accurate. The aim of the study is fulfilled. The Results are presented clearly and have been discussed sufficiently well.

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BRIEF ARTICLE

Pancreatic and pulmonary mast cells activation during experimental acute pancreatitis

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Abstract

AIM: To study the activation of pancreatic and pulmonary mast cells and the effect of mast cell inhibition on the activation of peritoneal and alveolar macrophages during acute pancreatitis.

METHODS: Pancreatitis was induced by intraductal infusion of 5% sodium taurodeoxycholate in rats. The mast cell inhibitor cromolyn was administered intraperitoneally (i.p.) 30 min before pancreatitis induction. The pancreatic and pulmonary tissue damage was evaluated histologically and mast cells and their state of activation were evaluated. Peritoneal and alveolar macrophages were obtained and the expression of tumor necrosis factor α was determined. Myeloperoxidase activity was measured to evaluate the effect of mast cell inhibition on the progression of the inflammatory process. Finally, the effect of plasma on cultured mast cells or macrophages was evaluated *in vitro*.

RESULTS: The mast cell stabilizer significantly reduced inflammation in the pancreas and lung and the activation of alveolar macrophages but had no effect on peritoneal macrophages. Mast cell degranulation was observed in the pancreas during pancreatitis but no changes were observed in the lung. Plasma from rats with pancreatitis could activate alveolar macrophages but did not induce degranulation of mast cells *in vitro*.

CONCLUSION: Pancreatic mast cells play an important role in triggering the local and systemic inflammatory response in the early stages of acute pancreatitis. In contrast, lung mast cells are not directly involved in the inflammatory response related to pancreatic damage.

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Key words: Cytokines; Inflammation; Macrophages; Mast cells; Pancreatitis

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INTRODUCTION

Acute pancreatitis represents a substantial clinical problem with increasing incidence and it is associated with high morbidity and mortality[1]. The most important predictor of mortality is the development of persistent or multiple organ failure and the commonest affected organ is the lung[2,3]. In these cases, acute lung injury is frequently related to early deaths in the first week of the disease[4]. The mechanisms involved in triggering distant organ inflammation are unclear, however, in addition to the release of activated hydrolytic enzymes, different pathways have been reported, including cytokines[5], oxygen-derived free radicals[6] or activated complement[7].

Among these mechanisms, mast cells have been reported to contribute to several aspects of pancreatitis-associated lung injury. These cells release a number of mediators, including histamine, tumor necrosis factor (TNF)α or monocyte chemotactic protein-1 (MCP-1) which could have a strong effect on pulmonary endothelial cells, thus potentiating the progression of inflammation[8,9]. The expression of different adhesion molecules increases early after pancreatitis induction, and in some of these molecules this increase could be prevented by administering mast cell degranulation inhibitors such as sodium cromolyn[10,11]. These observations suggest that masts cells are responding to mediators released during pancreatitis and, when activated, play a role in the induction of endothelial lung dysfunction and in the progression of the local and systemic inflammatory process.

Mast cells are usually located close to endothelial cells and this explains their effect on endothelial dysfunction when activated during pancreatitis. However, tissue-related variability on the number, phenotype and distribution of mast cell populations have been reported, resulting in different activation during inflammatory processes[12]. In the case of acute pancreatitis, the use of mast cell stabilizers prevented changes in systemic inflammation and in endothelial permeability in different organs[10], however, the involvement of the particular mast cell populations remains unclear.

In this work we have evaluated the effect of mast cell inhibition on the activation of peritoneal and alveolar macrophages in an experimental model of acute pancreatitis.

MATERIALS AND METHODS

Animal model of acute pancreatitis

Male Wistar rats (250-300 g b/w) (n = 6 each group) were anaesthetized with 10% urethane (1 mL/100 g, i.p.). The biliopancreatic duct was cannulated through the duodenum and the hepatic duct was closed by a small bulldog clamp. Severe acute pancreatitis was induced by retrograde infusion into the biliopancreatic duct of 5% sodium taurocholate (Sigma Chemical, St. Louis, MO, USA) in a volume of 0.1 mL/100 g b/w using a *Harvard* '22' infusion pump (Harvard Instruments, Edenbridge, UK)[13]. Control animals received an intraductal infusion of saline solution (0.9% NaCl). In a group of animals, cromolyn (Sigma, St Louis, MO, USA) (5 mg/kg b/w) was administered i.p. 30 min before pancreatitis induction. Three hours after induction, tissue samples of pancreas and lung were obtained, immediately frozen and maintained at -80℃ until processed. This time point was selected because we previously reported that, in this model, a significant systemic inflammation is initiated three hours after the induction of pancreatitis[14]. Plasma samples were pelleted and the supernatant was stored at -40℃ until use. Pancreas and lung samples were also obtained and stored for histological analysis.

Histological analysis

Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraplast, sectioned in 5 μm slices and stained with toluidine blue (0.1%). The dye was allowed to dry on the slide for a few seconds; the slides were then rinsed in xylene for 5-10 min. and rinsed twice with acetone. Finally, the slides were cleared in xylene and mounted in diphenylphthalein xylene. Sections were evaluated by light microscopic examination.

Cell culture

Peritoneal macrophages were harvested by 5 peritoneal washes with 10 mL of phosphate buffered saline (PBS) containing 3 units/mL heparin. The obtained cell suspension was centrifuged (300 × g, 7 min). Cells were suspended in the RPMI1640 culture medium containing 10% fetal calf serum, 2 mmol/L glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL). Aliquots of about 3×10^6 cells were plated in 6 well plates and cultured at 37℃ under a gas phase of air/CO_2 (95:5). After an attachment period of 4 h, the non-adhered cells were removed by shaking. The resulting adherent population consisted of > 92% peritoneal macrophages.

Alveolar macrophages were obtained by bronchoalveolar wash. After exsanguinations, lung and trachea were excised *en bloc* and washed 5 times with 10 mL cold (4℃) saline solution. The supernatant was centrifuged at 300 × g for 7 min and the cells were resuspended in RPMI1640 culture medium containing 10% fetal calf serum, 2 mmol/L glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL). Aliquots of about 3×10^6 cells were plated in 6 well plates and cultured at 37℃ un-

der a gas phase of air/CO_2 (95:5). After an attachment period of 4 h, the non-adhered cells were removed by shaking. The resulting adherent population consisted of > 95% alveolar macrophages.

The rat mast cell line RBL-2H3 was maintained as a monolayer culture in RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) in an incubator with 5% CO_2 at 37℃.

In vitro effect of plasma

The effects of circulating mediators on mast cells or macrophages were evaluated by incubating alveolar macrophages or the mast cell line RBL-2H3 with plasma obtained from controls, animals with pancreatitis or with cromolyn treated pancreatitis. Cells were cultured in 12 well plates in the presence of 20% plasma in the culture media. One hour after culture at 37℃, the levels of histamine were measured in RBL-2H3 supernatants. For macrophages, RNA was obtained and the expression of TNFα was evaluated by quantitative real-time polymerase chain reaction (RT-PCR).

RNA isolation and RT-PCR

Total RNA from cells was extracted using the TRizol® reagent (Invitrogen, Carlsbad, CA, USA). The RNA was quantified by measurement of the absorbance at 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA).

cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA), and reverse transcription was then performed on 1 μg RNA sample by adding iScript reagents. The reaction was incubated at 25℃ for 5 min, 42℃ for 30 min, and 85℃ for 5 min, and then stored at -80℃.

Subsequent PCR amplification was performed in a DNA Engine, Peltier Thermal Cycler (Bio-Rad Laboratories, CA, USA) using IQTM SYBR Green Super mix and the correspondent rat primers: TNFα forward: 5'-AACTCCCAGAAAAGCAAGCA-3' reverse: 5'-CGAGCAGGAATGAGAAGAGG-3'; GAPDH forward: 5'-CTGTGTCTTTCCGCTGTTTTC-3', reverse: 5'-TGTGCTGTGCTTATGGTCTCA-3'.

Initial denaturation was followed by 40 cycles of DNA amplification with fluorescence detection at the end of the elongation step (SYBR Green format). Reactions were performed in duplicate and threshold cycle values were normalized to GAPDH gene expression. The specificity of the products was determined by melting curve analysis. The ratio of the relative expression of target genes to GAPDH was calculated by using the ΔC(t) formula.

Lipase

Plasma lipase was determined using commercial turbidimetric assay kits from Randox (Antrim, UK), according to the supplier's specifications.

Histamine analysis

Histamine levels in plasma and in cell culture supernatants were evaluated using a commercial ELISA assay from Labor Diagnostika Nort (Nordhorn, Germany) according to the supplier's specifications.

TNFα

TNFα concentration in the cell culture medium was measured using a commercial kit for rat TNFα from BLK International (Badalona, Spain), according to the supplier's specifications.

Myeloperoxidase

Neutrophilic infiltration was assessed by measuring myeloperoxidase (MPO) activity. MPO was determined photometrically with 3,3',5,5'-tetramethylbenzidine as substrate. Tissue samples were homogenized with 0.5% hexadecyltrimethylammonium bromide in 50 mmol/L phosphate buffer at pH 6.0. Homogenates were disrupted for 30 s using a Labsonic sonicator (Braun Biotech, Inc., Allentown, PA, USA) at 20% power and submitted to three cycles of snap freezing in dry ice and thawing before a final 30 s sonication. Samples were incubated at 60℃ for 2 h and then spun down at 4000 × *g* for 12 min. The supernatants were collected for MPO assay. Enzyme activity was assessed photometrically using 630 nm wavelength. The assay mixture consisted of 20 μL supernatant, 10 μL tetramethylbenzidine (final concentration 1.6 mmol/L) dissolved in DMSO, and 70 μL H_2O_2 (final concentration 3.0 mmol/L) diluted in 80 mmol/L phosphate buffer, pH 5.4. The results are expressed as units (U) MPO activity per g protein.

Protein measurement: Total protein concentration in homogenates was determined using a commercial kit from BioRad (Munich, Germany).

Statistical analysis

Data are expressed as mean ± SE. Means of different groups were compared using a one-way analysis of variance. Tukey's multiple comparison test was performed to evaluate significant differences between groups. Differences were assumed to be significant when $P < 0.05$.

RESULTS

Mast cell activation during pancreatitis

Histological analysis revealed the presence of mast cells in the interlobular areas of control pancreas (Figure 1A) and a general pancreatic mast cell degranulation after induction of pancreatitis (Figure 1B). This degranulation was prevented by cromolyn treatment (Figure 1C). In lungs, mast cells could also be observed (Figure 1D), however, no apparent degranulation was detected in histological samples of the lung 3 h after induction of pancreatitis (Figure 1E). Cromolyn treatment had no effect on lung mast cells (Figure 1F).

Effects of mast cell inhibition

Pancreatitis resulted in increased levels of circulating lipase and histamine in plasma as well as enhanced MPO activity in both pancreas and lung (Figure 2). The inhibition of mast cell degranulation with cromolyn did not

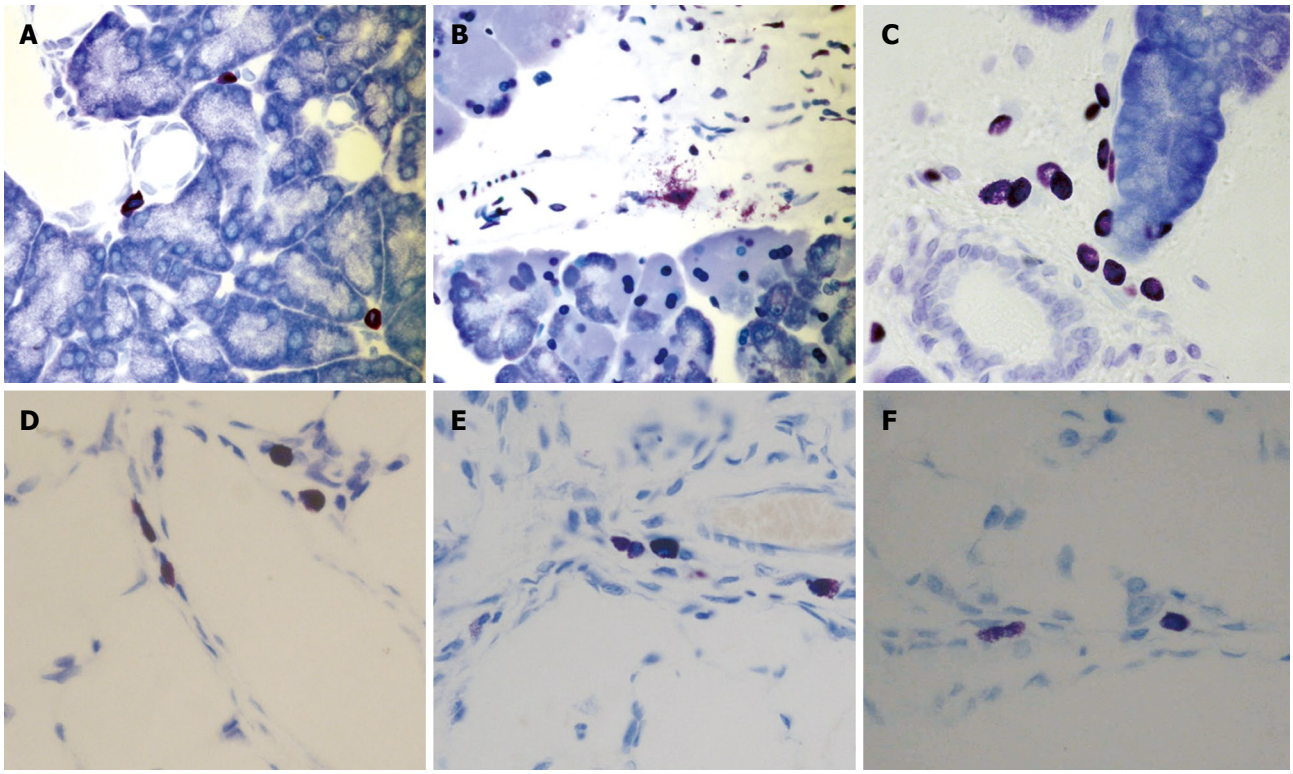

Figure 1 Presence of mast cells in pancreas (A-C) and lung (D-F), in control (A and D). Three hours after induction of pancreatitis (B and E) and under cromolyn treatment (C and F). Degranulating mast cells were observed in the pancreas after pancreatitis induction (B). Cromolyn treatment prevented mast cell degranulation (C). In contrast, no evident degranulation was observed in lung after induction of pancreatitis. Toluidine blue, × 40.

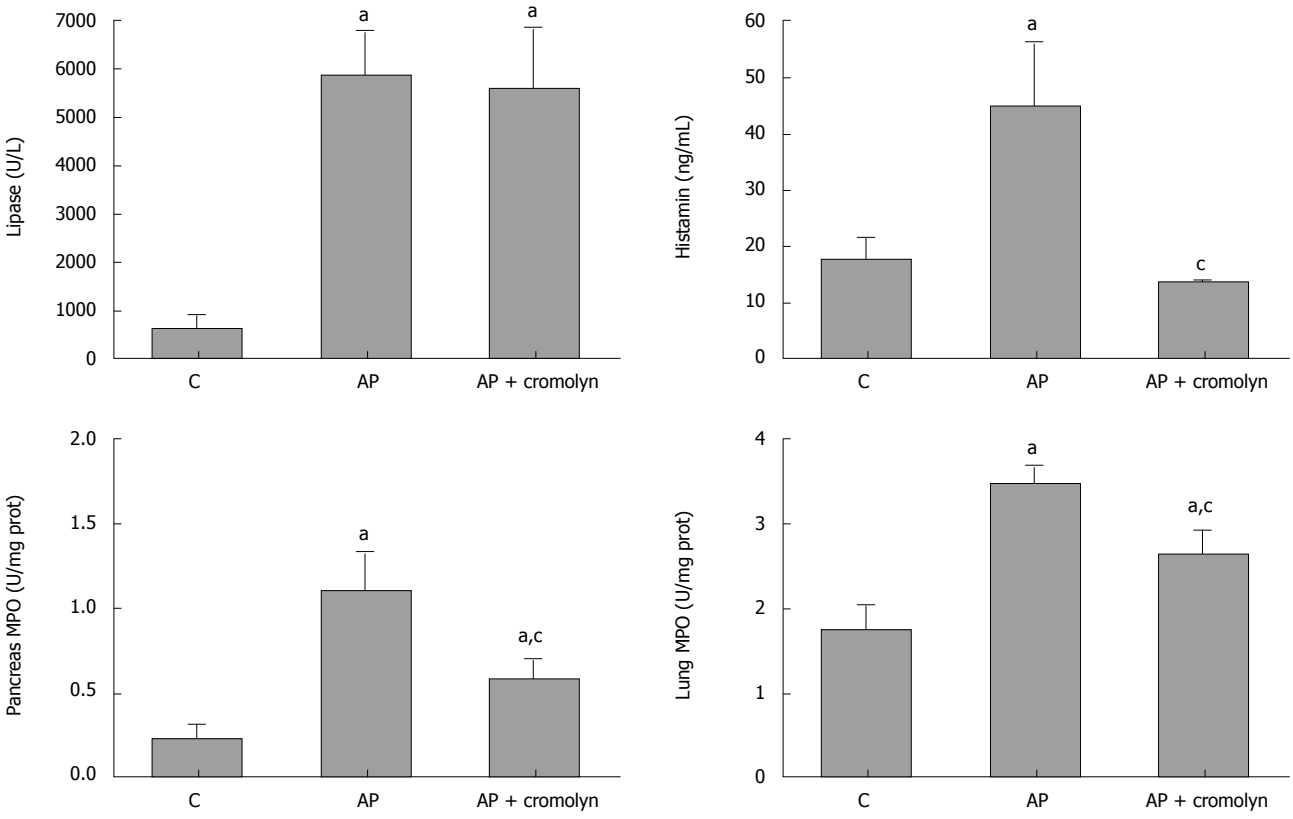

Figure 2 Effect of mast cell inhibitor, cromolyn. Three hours after pancreatitis induction, increased levels of lipase and histamine were detected in plasma. Cromolyn treatment had no effect on lipase, which is related to acinar cell damage, but prevented the increase in histamine. In tissue, leukocyte infiltration was evaluated by measuring myeloperoxidase (MPO) activity. Pancreatitis resulted in increased MPO activity in both pancreas and lung. Cromolyn treatment partially prevented these increases. $^{a}P < 0.05$ *vs* C; $^{c}P < 0.05$ *vs* AP. C: Control; AP: Acute pancreatitis.

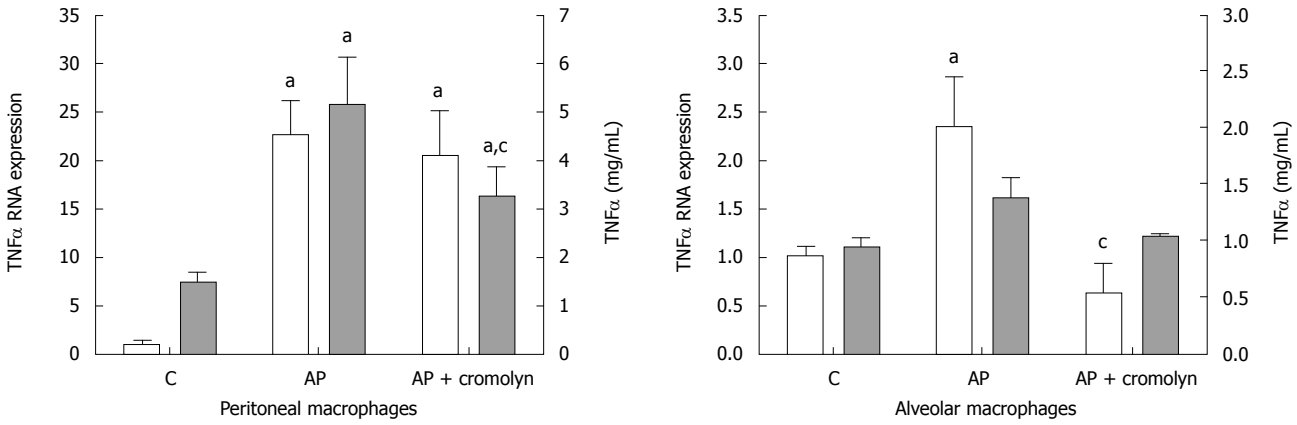

Figure 3 Both peritoneal and alveolar macrophages were activated after induction of pancreatitis, but the expression of tumor necrosis factor α mRNA in peritoneal macrophages was one order of magnitude higher than that observed in alveolar macrophages. Tumor necrosis factor (TNF)α release was induced in peritoneal cells, while in alveolar cells the observed increase was not statistically significant. Cromolyn treatment completely prevented the activation of alveolar macrophages. In contrast, peritoneal macrophages remained activated under cromolyn treatment. $^{a}P < 0.05$ *vs* C; $^{c}P < 0.05$ *vs* AP. C: Control; AP: Acute pancreatitis.

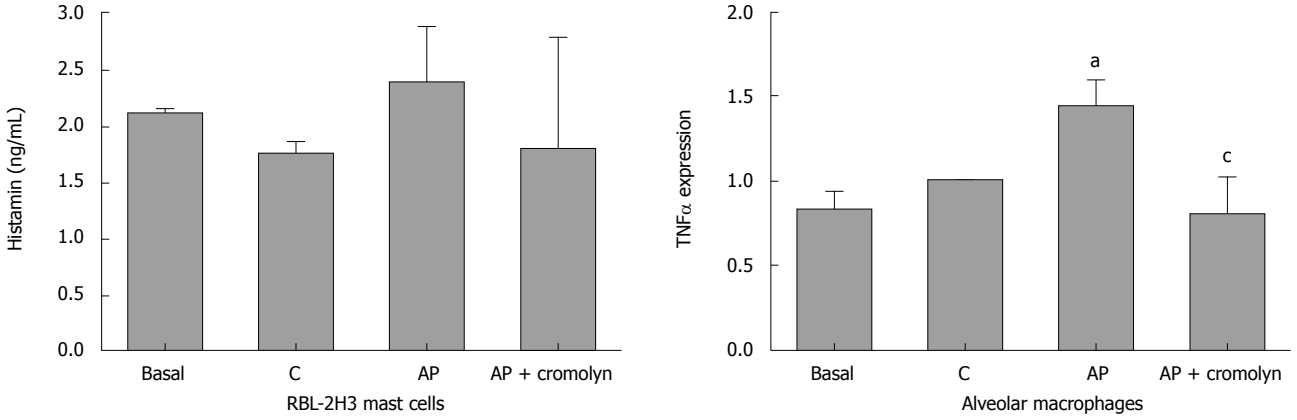

Figure 4 The effect of plasma on cultured mast cell line RBL-2H3 and alveolar macrophages. Mast cells were not activated by plasma from animals with pancreatitis. In contrast, the expression of tumor necrosis factor (TNF)α in alveolar macrophages was induced by plasma from animals with pancreatitis. This induction was not observed when animals were treated with cromolyn. $^{a}P < 0.05$ *vs* C; $^{c}P < 0.05$ *vs* AP. C: Control; AP: Acute pancreatitis.

modify the lipase levels, but resulted in a reduction in pancreatic MPO activity, indicating that this treatment had no effect on acinar cell damage but reduced pancreatic inflammation. Inhibition of the inflammatory process in lung was also observed. Nevertheless, these inhibitions did not achieve the control values and MPO activity remained significantly increased with respect to the control group in both pancreas and lung.

Changes in peritoneal and alveolar macrophages

Pancreatitis resulted in the induction of TNFα expression in both peritoneal and alveolar macrophages (Figure 3). However, at this time point the activation observed in peritoneal macrophages was one order of magnitude greater than that observed in alveolar macrophages. This was reflected in the release of TNFα which only showed a significant increase in peritoneal macrophages after pancreatitis induction, while the increase observed in alveolar macrophages was not statistically significant (Figure 3). Inhibition of mast cell degranulation had no effect on peritoneal macrophage activation, but completely prevented the increase observed in TNFα expression in alveolar macrophages.

Effect of plasma on cultured mast cells and macrophages

Incubation of the RBL-2H3 cell line with plasma obtained from animals with pancreatitis did not result in increased histamine release (Figure 4). In contrast, this plasma was able to induce the activation of alveolar macrophages, reflected in an increase in the expression of TNFα. This induction was not observed when plasma was obtained from animals treated with cromolyn (Figure 4).

DISCUSSION

The development of systemic inflammation during the progression of severe acute pancreatitis involves multiple pathways and cell systems. Among them, mast cells appear to play a pivotal role between the hydrolytic enzymes released by damaged pancreatic acinar cells and the stress-induced response mediated by free radicals and inflammatory mediators.

In this sense, mast cells seem to play a role amplifying the acute inflammatory response in different organs early after the onset of pancreatitis. Several mediators known to be released by mast cells, including platelet activating factor (PAF), histamine or prostaglandin D_2 (PGD_2), have been shown to be increased a few minutes after the induction of pancreatitis in experimental models[15,16]. In addition, the administration of mast cell inhibitors results in a reduction of the local and systemic inflammatory response and, in particular, prevents changes in endothelial cells and vascular permeability[10].

However, it is important to evaluate the particular role of the different mast cell populations in this process, in order to design therapeutic strategies centered on these cells. Due to the rapid activation of pancreatic mast cells in the onset of pancreatitis, the obvious therapeutic target may be pulmonary mast cells that are suspected of being activated in the later stages of the disease.

In the present study, we evaluated the degranulation of mast cells in pancreas and lung and found a different response during pancreatitis. Histological evaluation showed a clear and extensive degranulation of mast cells located in pancreatic tissue (Figure 1B). As expected, this degranulation was prevented by cromolyn administration (Figure 1C). In contrast, no clear evidence of mast cell degranulation was observed in lung tissue (Figure 1E).

This was a surprising result, taking into account that cromolyn administration resulted in a clear reduction in lung inflammation revealed by MPO activity and by a lower activation of alveolar macrophages (Figure 3). In addition, these results are in line with other authors who reported on the critical role of mast cells in the inflammatory response in lung during pancreatitis.

An explanation for this apparent contradictory result is an indirect effect of pancreatic mast-cell derived mediators on distant organs. Activation of mast cells results in the immediate release of mediators that play a role in the activation of circulating leukocytes as demonstrated by Zhao *et al*[11]. On the other hand, the progression of inflammation in pancreatic tissue is modified by cromolyn treatment (Figures 1 and 2). Consequently, it is suspected that the profile of pro-inflammatory mediators released to the bloodstream by pancreatic tissue and their ability to induce lung endothelial dysfunction could be modified by pancreatic mast cell inhibition.

To evaluate this possibility we treated alveolar macrophages as well as the mast cell line RBL-2H3 *in vitro* with plasma obtain from the different experimental groups. Our results indicate that plasma from the pancreatitis-induced group did not stimulate the production of significant amounts of histamine in culture (Figure 4). This result suggests that while pancreatic damage could enhance the activation and degranulation of mast cells in the pancreas a few minutes after pancreatitis induction[8], mediators present in plasma are not sufficient to activate these cells in distant organs.

However, plasma from pancreatitis animals was able to induce the activation of macrophages *in vitro*, reflected in the increased expression of TNFα. This effect was clearly reduced when animals were treated with cromolyn (Figure 4). Together, these results indicate that pancreatic mast cells play an important role in triggering the local and systemic inflammatory response in the early stages of acute pancreatitis. In contrast, lung mast cells are not directly involved in the inflammatory response related to pancreatic damage. The early activation reported in pancreatic mast cells may make the use of these cells as a pharmacological target difficult due to the short therapeutic window.

COMMENTS

Background

Mast cells have been reported to contribute to several aspects of pancreatitis associated lung injury. However, the involvement of particular mast cell populations remains unclear.

Research frontiers

Using an experimental model of acute pancreatitis in rats, the authors evaluated the activation of mast cells from pancreas and lung as well as the effect of mast cell inhibitors on progression of the inflammatory reaction.

Innovations and breakthroughs

Pancreatic mast cells play an important role in triggering the local and systemic inflammatory response in the early stages of acute pancreatitis. In contrast, lung mast cells are not directly involved in the systemic inflammatory response related to pancreatic damage.

Applications

The identification of active mast cells in the early stages of pancreatitis may improve our understanding of their role in this disease and the possible therapeutic strategies focussed on these cells.

Terminology

Cromolyn is a mast cell stabilizer that prevents the release of inflammatory mediators, such as histamine, from these cells.

Peer review

In this work, the authors have evaluated the effect of mast cell inhibition on the activation of peritoneal and alveolar macrophages in an experimental model of acute pancreatitis. The manuscript portrays a good effort by its authors.

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BRIEF ARTICLE

Double balloon enteroscopy examinations in general anesthesia

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Abstract

AIM: To demonstrate that the double balloon enteroscopy (DBE) can be safely performed in general anesthesia with intubation.

METHODS: We performed a retrospective examination between August 2005 and November 2008 among patients receiving intubation narcosis due to DBE examination. The patients were grouped based on sex, age and physical status. Anesthesia records included duration of anesthesia, quantity of medication used and anesthesia-related complications. We determined the frequency of complications in the different groups and their relation with the quantity of medication used and the duration of anesthesia.

RESULTS: We compiled data for 108 cases of general anesthesia with intubation. We did not observe any permanent anesthesia-related complications; the most frequent side effects of anesthesia were hypotension (30.55%), desaturation (21.29%), and apnea (17.59%). These complications were significantly more frequent among patients with multiple additional diseases [hypotension (23.1% *vs* 76.9%, $P = 0.005$), desaturation (12.3% *vs* 69.2%, $P < 0.001$) and apnea (7.7% *vs* 53.8%, $P = 0.001$)], however, their incidence was not proportional to the quantity of medication used or the duration of anesthesia.

CONCLUSION: General anesthesia with intubation is definitely a viable option among DBE methods. It is highly recommended in patients with multiple additional diseases.

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Key words: Double balloon enteroscopy; General anesthesia; Intubation; Sedation; Patient autonomy

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INTRODUCTION

Although gastroenterological endoscopic examinations are performed with some form of sedation or anesthesia at increasing rates worldwide, gastroscopy and colonoscopy are still often performed without any sedation, even today[1]. A reason for the widespread use of anesthesia is that patients receiving sedation are more satisfied, because they recall less pain and discomfort related to the intervention. Also, gastroenterology specialists

can examine patients that are otherwise not suitable for examination because of psychological reasons or strong abdominal pain.

However, the dissemination of gastroenterology sedation has limitations[2]. This is partly because the intervention costs significantly more because of personnel and infrastructure requirements, and also because anesthesia itself may also have moderate or severe side effects[3]. According to the literature, over 50% of the complications are heart- or lung-related (aspiration, airway obstruction, low ventilation frequency, vaso-vagal episode, oversedation). So far, there is no consensus on whether sedation should be performed by gastroenterology specialists, anesthetist physicians or assistants, or the patient (patient-controlled anesthesia); or whether the medication should be administered as a bolus, as a continuous infusion, or automatically provided based on pre-calculated plasma level (target controlled infusion)[4].

The emergence of double balloon enteroscopy (DBE) among endoscopic examinations also means a shift of paradigm for internal medicine specialists, because its safe and efficient completion requires an advanced level of anesthesia. As the method has only been widely used for a couple of years, little data are available on the respective anesthetic procedures. According to the literature, three methods of sedation are used with significant geographical preferences, including conscious sedation, deep sedation (propofol anesthesia) and general anesthesia.

The goal of our research was to assess the suitability and advantages of general anesthesia with intubation for DBE.

MATERIALS AND METHODS

Patients

We retrospectively analyzed the data from 108 patients that had not been pre-selected, in whom DBE was carried out under general anesthesia with intubation. The interventions were carried out in the 1st Department of Internal Medicine of Semmelweis University, Budapest, Hungary between August 2005 and November 2008. Patients were classified into groups based on sex, age, physical status (ASA Physical Status Classification System) and DBE indication[5]. Anesthesia records included the duration of the intervention, anesthesia protocol, quantity of medication used, and complications.

Following recovery from anesthesia, the patients were asked to recall memories of the intervention and describe any possible complaint.

Method of anesthesia

Electrocardiography and transdermal oxygen saturation were constantly monitored during the intervention, and non-invasive blood pressure measurements were also performed every 5 min. Based on the literature, the definitions were as follows: hypotension, systolic blood pressure < 90 mmHg; desaturation, transdermal oxygen saturation < 90%; and apnea, > 30 s pause in respiration.

During intervention, proper anesthesia was provided by the combined administration of benzodiazepine, opioids and propofol in all cases; the mentioned medications were selected based on availability because these are all readily available in every endoscopy laboratory. We supposed that complete anesthesia was reached with their combined usage, and we also wished to adapt continuously the degree of anesthesia to the requirements of the intervention.

First, peripheral venous access was provided, and then infusion was administered (500-1000 mL). All patients received 0.5 mg atropine prior to the intervention, followed by gradual intravenous midazolam injection (3-10 mg) to reach a consciousness level equivalent to conscious sedation. All patients received 1-1.5 μg/kg fentanyl, and induction of narcosis was achieved by 1 mg/kg propofol as a bolus. For the maintenance of narcosis, further doses of propofol were used. We used two anesthesia protocols. According to these, propofol was either provided as continuous infusion or given in discrete fractions. In the case of continuous use, the infusion rate was set at 200 mg/h; for fractioned use, 25 mg fractions were given as a bolus following induction and intubation, until the end of intervention. If the degree of anesthesia was insufficient (patient motion, changes in vegetative reactions), we increased the speed of propofol infusion, or another fraction was administered. Fentanyl was repeatedly provided every 30 min at 0.5-1 μg/kg.

Statistical analysis

Arithmetic mean and SD values were used for continuous parameters, whereas frequency percentages were calculated for discrete parameters. Statsoft version 8.0 software (www.statsoft.com) was used for statistical analysis. The quantity of medication was compared using non-parametric variance analysis (Kruskal-Wallis analysis of variance), and the frequency of the observed complications was compared with Fisher's exact test among the various groups. $P < 0.05$ was considered statistically significant.

RESULTS

The indications for intervention in the 108 patients enrolled in the study are presented in Table 1. In patients with obscure gastrointestinal bleeding (OGIB), abnormal small-bowel findings were seen in 41 patients (65.1%). Most of them were classified as probable (angiodysplasia, erosion), and others as definitive (e.g. small ulcers) causes of bleeding. Other definitive causes were malignant disease, found in five patients, including polypoid gastrointestinal stromal tumor (GIST) in three patients, non-Hodgkin lymphoma (NHL) in one, and melanoma in one. In suspected inflammatory bowel disease (IBD), enteroscopy confirmed the diagnosis in five out of 12 cases. In patients with suspected neoplasia/stenosis, malignant disease was proven in three cases. In patients with known polyposis syndromes [familial adenomatous polyposis (FAP) or Peutz-Jeghers syndrome], small-bowel polyps were removed in eight patients. The average insertion length was 209 cm (50-460 cm, SD: 113 cm). Using the oral route (n = 95), a larger proportion of the

Table 1 Indications for double balloon enteroscopy

	n (%)
Suspected malignancy/stenosis	8 (7.4)
OGIB	63 (58.3)
Peutz-Jeghers syndrome	5 (4.6)
Polyposis	6 (5.6)
Angiodysplasia	6 (5.6)
IBD	12 (11.1)
Chronic cramping pain	6 (5.6)
Unknown fever or loss of weight	1 (0.9)
Irritable bowel syndrome	1 (0.9)

DBE: Double balloon enteroscopy; OGIB: Obscure gastrointestinal bleeding; IBD: Inflammatory bowel disease.

Table 2 Demographic and clinical data of patient groups

	P1 (SD)	P2 (SD)	P3 (SD)	Sum (SD)
n	65	30	13	108
Age (yr)	45.88 (15.93)	60.42 (13.36)	70.69 (19.47)	52.53 (18.44)
Duration (min)	91.85 (24.79)	79.17 (19.17)	65.77 (10.77)	85.18 (23.72)
Propofol (mg)	464.31 (91.84)	410.33 (66.82)	346.92 (61.29)	435.18 (91.16)
Midazolam (mg)	7.17 (1.29)	5.50 (1.04)	4.08 (0.76)	6.31 (1.60)
Fentanyl (mg)	0.1307 (0.0350)	0.1217 (0.0284)	0.0731 (0.0259)	0.1213 (0.0369)

small intestine was accessible for examination (226 cm, SD: 107 cm) compared with procedures that started with anal endoscope insertion and colonoscopy (n = 13, 98 cm, SD: 58 cm, $P < 0.01$).

Fifty-five patients were male (50.92%) and 53 were female (49.08%), with the average age being 52.53 years (SD: 18.44 years). The patients were classified into three groups based on the ASA Physical status classification system (ASA P1-P3). P1 included 65 patients (average age: 45.87 years, SD: 15.93 years), P2 included 30 patients (average age: 60.42 years, SD: 13.36 years), and P3 included 13 patients (average age: 70.69 years, SD: 19.47 years). The three groups were compared based on the duration of the intervention, the quantity of medication used, and the observed complications. Demographic and clinical data of the three groups are shown in Table 2.

The average length of the intervention was 85.18 min (SD: 23.72 min); with 91.85 min in P1 (SD: 24.79 min), 79.17 min (SD: 19.17 min) in P2, and 65.77 min (SD: 10.77 min) in P3. Although the time of intervention gradually decreased with deteriorating physical status, a significant difference was only found between P1 and P3 ($P < 0.001$).

The average amount of propofol used during the intervention was 435.18 mg (SD: 91.16 mg) per patient, with 464.31 mg (SD: 91.84 mg) in P1, 410.33 mg (SD: 66.82 mg) in P2, and 346.92 mg (SD: 61.29 mg) in P3. The dose of propofol decreased in patients with deteriorating physical status and significant differences were found between groups P1 and P2 ($P = 0.027$) and P1 and P3 ($P < 0.001$).

Table 3 Number and ratio of frequent complications *n* (%)

	P1	P2	P3	Sum
n	65	30	13	108
Hypotension	15 (23.1)	8 (12.3)	10 (76.9)	33 (30.6)
Desaturation	8 (12.3)	6 (20.0)	9 (69.2)	23 (21.3)
Apnea	5 (7.7)	7 (23.3)	7 (53.8)	19 (17.6)

The average quantity of midazolam used per patient was 6.31 mg (SD: 1.60 mg); with 7.14 mg (SD: 1.29 mg) in P1, 5.5 mg (SD: 1.04 mg) in P2, and 4.08 mg (SD: 0.76 mg) in P3. Significant differences were found between the groups P1 and P2 ($P < 0.001$) and P1 and P3 ($P < 0.001$), as well as P2 and P3 ($P = 0.045$). The average amount of fentanyl used per patient was 0.1213 mg (SD: 0.0369 mg); with 0.1307 mg (SD: 0.0350 mg) in P1, 0.1217 mg (SD: 0.0284 mg) in P2, and 0.0731 mg (SD: 0.0259 mg) in P3. Significant differences were found between groups P1 and P3 ($P < 0.001$) and P2 and P3 ($P = 0.001$).

Among anesthesia-related complications recorded during the intervention, hypotension, desaturation and apnea occurred frequently. Table 3 presents the number of complications and their comparison between the groups. We analyzed statistically the correlation between the occurrence of the above complications (hypotension, desaturation and apnea) and patients' physical status, the duration of the intervention, and the quantity of medication (propofol, midazolam, fentanyl).

Only the physical-status-based classification and the occurrence of the recorded complications showed a significant positive correlation. ASA P stage significantly influenced the frequency of hypotension ($P = 0.005$), de-saturation ($P < 0.001$) and apnea ($P < 0.001$). These complications were more frequently observed among patients classified into group P3 than would have been expected based on random incidence.

A significant positive correlation was not found between the quantity of medication and complications. There was a significant negative correlation between propofol dosage and the development of hypotension ($P = 0.002$). We found a significant negative correlation between midazolam dosage and the three most frequent complications (hypotension, $P = 0.001$; de-saturation, $P = 0.004$; apnea, $P = 0.001$). There was a significant negative correlation between fentanyl dosage and desaturation ($P = 0.003$), but not with the other complications. There was a significant negative correlation between the duration of the intervention and the frequency of desaturation ($P = 0.018$) and apnea ($P = 0.040$).

Among the 108 DBE anesthesia cases, three imminent anesthesia-related problems had to be resolved. In one case, peripheral venous access could not be provided due to the physical status of the patient, but instead, central venous access was established without any complication. In another case, intubation could not be completed and hence enteroscopy had to be delayed. One week later, both the intubation and intervention were completed with

the application of a depolarizing muscle relaxant. For a third patient, who had obesity and chronic obstructive pulmonary disease, continuous respiration assistance and oxygen supply had to be provided, and extubation could only be performed in the seated position due to breathing difficulty.

Anesthesia was not related to any permanent or severe complication (aspiration, malignant dysrhythmia, resuscitation, malignant hyperthermia) in any case. More than 98% of the patients had amnesia concerning events during anesthesia. Frequent complaints included discomfort at the site of peripheral venous access, sore throat or dysphagia, and abdominal distension.

DISCUSSION

Thanks to international recommendations based on the accumulating amount of data published about sedation techniques related to endoscopic interventions performed in the gastrointestinal tract, these interventions have become extremely safe[6-8]. Severe complications are generally rare and deadly fatal complications mostly affect patients in a severely impaired or terminal physical state[9]. Data concerning recently introduced enteroscopic examinations and the related sedation techniques are scarce, and randomized, multicenter comparative studies with large numbers of patients have been lacking.

The goal of our study was to examine the utility of general anesthesia with intubation as a method of choice for DBE. The enrolled patients were divided into three groups according to the ASA Physical Status Classification System.

We first examined whether the 108 enteroscopy cases corresponded with published data in terms of intervention indications and duration. Among the indications, OGIB (58.33%), IBD (11.11%) and tumor (7.41%) were the most frequent in our practice, as in the literature (OGIB: 59%-62.8%; IBD: 2.9%-6.4%; tumor: 8.3%-10.2%)[10-12]. The average duration of the intervention in our study (85.18 min) was also found to be similar to that in the literature (53-113 min)[13,14]. The detailed outcome of the endoscopic procedures has been published in a separate paper[15].

However, we need to highlight some differences in sedation complications. Anesthesia-related complications occurred at much higher frequencies in our practice than during conscious sedation described in the literature, but these have either quickly resolved without any or with minor medical intervention. Hypotension was found to be the most frequent complication in our study (30.55%), which occurred at much lower frequencies during conscious sedation (1.8%-23.08%)[10,13,16]. If hypotension was observed, we increased intravenous fluid therapy, although the positive effects of the procedure are not obvious[17], and we also decreased the administration of propofol and fentanyl. Hypertensive drugs were not used in any case, and hypotension resolved within minutes with the above procedures.

The frequency of desaturation was 21.29% in our study, which is similar to the frequencies reported in the literature (0%-30.78%)[13,18]. In cases of hypoxia, transient or continuous oxygen inhalation was necessary, depending on the patient's requirements, and if oxygen levels normalized, we discontinued oxygen administration. Apnea was observed in 17.59% of the cases in which respiratory assistance was initiated, which was discontinued as soon as spontaneous respiration was restored. However, some patients required continuous respiratory assistance during the intervention. The severe complication of aspiration did not occur during intubation, and probably this is the most prominent difference between intubation and conscious sedation (0% *vs* 1.2%-2.77%).

We found a significant positive correlation between the number of complications and poor physical status. Poor physical status and senior age both predict occurrence of hypotension, desaturation and apnea. With the increase in ASA physical status level, the duration of the intervention and the quantity of medication decreased.

We consider it important to highlight that side effects observed during anesthesia are not related to medication use, because there was no significant positive correlation found between the frequency of complications and dose of propofol, midazolam or fentanyl. For some complications, the opposite was true, as patients with poor health status (P3), who experienced the most complications, received much less anesthetic.

The amount of medication used for narcosis in our study was higher than that required for examinations performed under conscious sedation[13]. The reason for this is that a greater amount of medication is required for deeper sedation (general anesthesia). Also, patients receiving orotracheal intubation require more medication to tolerate the procedure.

The ratio of complete amnesia observed among patients examined in intubation narcosis was much higher (98%) than among those receiving venous sedation (24%-56%)[14].

Therefore, who is advised to undergo general anesthesia as an alternative sedation method performed by an anesthetist? Every patient who belongs to a sedation-related risk group. Such risk factors include emergency interventions; senescence; cardiac, lung, renal or liver diseases possibly resulting in organ failure; pregnancy; drug or alcohol abuse; disorientation; post-prandial or non-cooperative patients, and alleged airway obstruction[4]. In our study, patients classified as P3 or higher also belonged to the high-risk group, therefore, ASA physical status helps us to choose the right method of anesthesia.

General anesthesia with intubation was a viable option when performing DBE in all three patient groups. With the deterioration of physical status (increasing ASA P status), the advantage of intubation narcosis increases compared to other sedation methods. In the case of poor physical status, the number of complications significantly increases, however, these are readily treatable due to pre-existing intubation. The occurrence of hypoxia, apnea or aspiration can result in an emergency situation in patients sedated without intubation, which can lead to deterioration of the patient's physical status and halt the course of

the examination, thus significantly increasing the number of complications and healthcare costs. General anesthesia can also be used safely in ASA groups P1-2 because severe or permanent anesthesia-related complications have not been observed in any case. Alternative anesthetic methods that suit the patient's needs will be justified in the future if, in the institutions performing enteroscopy, venous sedation (conscious or deep) and general anesthesia are provided. Self autonomy of patients with good health status, who are suitable for ambulatory intervention (ASA P1-2), should be emphasized, and after providing sufficient information, choices of alternative anesthesia methods should be offered.

Our study had some limitations, because the study was retrospective and patients were not randomized. Patient numbers were not equal between the groups, with especially few patients in group P3. Another limitation was that we performed only general anesthesia with intubation; the other sedation methods that were used for comparison were based on published data only. We compared the average frequency of side effects observed in our patients with those reported in the literature.

COMMENTS

Background

Three methods of sedation are used for double balloon enteroscopy (DBE), with significant geographical preferences, including conscious sedation, deep sedation (propofol anesthesia) and general anesthesia. The goal of this research was to assess the suitability and advantages of general anesthesia with intubation for DBE.

Research frontiers

The best anesthetic method for DBE is not clear at present, because all sedation methods have many different advantages and disadvantages.

Innovations and breakthroughs

This research shows that the ASA physical status classification system helps us to choose the right anesthetic method. With the deterioration of physical status (increasing ASA P status), the advantage of intubation narcosis increases compared to other sedation methods. The authors found a significant positive correlation between the number of complications and poor physical status. They consider that the side effects observed during anesthesia are not related to medication use.

Applications

Based on the results of this clinical study, general anesthesia with intubation was a viable option for DBE in all three patient groups. Alternative anesthetic methods that suit the patient's needs will be justified in the future in institutions that perform enteroscopy.

Peer review

The study demonstrates that the double balloon enteroscopy examination can also be safely performed in general anaesthesia with intubation.

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BRIEF ARTICLE

Does bilioenteric anastomosis impair results of liver resection in primary intrahepatic lithiasis?

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Abstract

AIM: To evaluate the long-term results of liver resection for the treatment of primary intrahepatic lithiasis. Prognostic factors, especially the impact of bilioenteric anastomosis on recurrence of symptoms were assessed.

METHODS: Forty one patients with intrahepatic stones and parenchyma fibrosis/atrophy and/or biliary stenosis were submitted to liver resection. Resection was associated with a Roux-en-Y hepaticojejunostomy in all patients with bilateral stones and in those with unilateral disease and dilation of the extrahepatic biliary duct (> 2 cm). Late results and risk factors for recurrence of symptoms or stones were evaluated.

RESULTS: There was no operative mortality. After a mean follow-up of 50.3 mo, good late results were observed in 82.9% of patients; all patients submitted to liver resection alone and 58.8% of those submitted to liver resection and hepaticojejunostomy were free of symptoms (P = 0.0006). Patients with unilateral and bilateral disease showed good late results in 94.1% and 28.6%, respectively (P < 0.001).

CONCLUSION: Recurrence of symptoms in patients with hepaticojejunostomy showed that this may not be the ideal solution. Further studies are needed to establish the best treatment for patients with bilateral stones or unilateral disease and a dilated extrahepatic duct.

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Key words: Biliary lithiasis; Bilioenteric anastomosis; Cholangitis; Intrahepatic lithiasis; Liver resection

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INTRODUCTION

Primary intrahepatic lithiasis or hepatolithiasis, is a prevalent disease in Southeastern Asia but is rare in the Western world; it is a challenging condition due to its varied forms of presentation and complex treatment. In some Western countries, it has been increasingly diagnosed and a relative incidence of 2.1% from all cases of biliary stone disease has been reported[1,2]. The goals of treatment are to promote stone clearance, control bile infection, decompress the biliary tree, and prevent progressive hepatic dysfunc-

tion. Since each patient has a distinctive stone distribution within the biliary tree, treatment has to be individualized accordingly. Liver resection has been reported to promote excellent long-term results, since stones and biliary strictures can be simultaneously removed reducing the risk of recurrence. In patients with unilateral stones, liver resection is considered a potentially curative treatment[3-8]. For bilateral stones, the ideal treatment has not yet been established; bilioenteric anastomosis or a percutaneous approach associated or not with liver resection have been employed with good long-term results in up to two thirds of cases.

Although resection can lead to a cure in patients with bilateral disease, the recurrence of symptoms is not rare. Moreover, it has been shown that patients submitted to liver resection associated with a bilioenteric anastomosis, had higher rates of recurrent cholangitis when compared to those submitted to resection only[4,8].

The purpose of this study is to report our experience with patients submitted to liver resection for the treatment of non-oriental hepatolithiasis, and to evaluate the influence of different prognostic factors, especially bilioenteric anastomosis, on late results.

MATERIALS AND METHODS

Ninety eight patients with symptomatic primary intrahepatic lithiasis were treated at our institution between 1990 and 2006.

According to our treatment protocol, liver resection was indicated in patients with irreversible hepatic lesions such as unilateral or segmental liver fibrosis/atrophy or the presence of intrahepatic biliary stenosis. A complementary Roux-en-Y hepaticojejunostomy was performed in patients with unilateral liver disease who presented with common bile duct stones with a duct diameter larger than 2 cm, and in all patients with bilateral stones[2].

Forty one patients (41.8%) underwent liver resection; data regarding gender, age, history of cholangitis and previous biliary surgery, intrahepatic stone location, liver function tests, intraoperative findings, type of surgery performed and postoperative outcome are presented in Table 1.

There were 16 men (39.9%) and 25 women (60.1%), with a mean age of 41.3 years (range 18 to 67 years). A history of right upper quadrant pain was present in all cases, jaundice in 31 patients (75.6%), cholangitis in 25 (61%) and nineteen (46.3%) had previously undergone biliary tract surgery: cholecystectomy in 13, hepaticojejunostomy in 3 and cholecystectomy plus common bile duct exploration in 3. None of the patients showed any sign of liver failure at physical examination.

Preoperative diagnosis was based on ultrasonography, helicoidal three-phase tomography, endoscopic or percutaneous cholangiography that in the last 5 years were replaced by magnetic resonance cholangiography. A complementary operative cholangiography was performed in all cases.

Indications for liver resection were: parenchymal atrophy in 27 patients, intrahepatic biliary stenosis in 8 and unilobular severe liver fibrosis in 6. Two patients were submitted to liver resection in a septic condition, due to cholangitis.

Table 1 Analysis of the effect of each variable on late results

Variable (*n*)	Late complications (poor results) *n* (%)	Statistical analysis
Gender		
Female (25)	6 (24.0)	$P = 0.1406$
Male (16)	1 (6.3)	
Previous biliary surgery		
No (22)	3 (13.6)	$P = 0.5291$
Yes (19)	4 (21.1)	
History of cholangitis		
Yes (25)	7 (28.0)	$P = 0.0608$
No (16)	0 (0)	
Preoperative serum bilirubin		
Normal (32)	6 (18.8)	$P = 0.5905$
Raised (9)	1 (11.1)	
Preoperative white blood cells		
Normal (37)	6 (16.2)	$P = 0.6574$
Raised (4)	1 (25.0)	
Stone location		
Unilateral (34)	2 (5.9)	$P < 0.0001$
Bilateral (7)	5 (71.4)	
Type of surgery		
Liver resection (24)	0 (0)	$P = 0.0006$
Liver resection + HJ (17)	7 (41.2)	
Major liver resection (more than 3 segments)		
Yes (14)	1 (7.1)	$P = 0.2237$
No (27)	6 (22.2)	

HJ: Hepaticojejunostomy.

Mean follow-up was 50.3 mo, ranging from 18 to 198 mo. Long-term results were considered good when no recurrence of symptoms or complications of the disease such as cholangitis or liver abscess during the follow-up period were observed.

Independent variables and their impact on late prognosis were compared using Student's t and Pearson's χ^2 tests. Statistical significance was set at $P < 0.05$.

RESULTS

Forty one patients were submitted to liver resection, 34 (82.9%) had unilateral disease and the left lobe was more frequently affected (28 cases). Bilirubin, alkaline phosphatase and gamma glutamyl transpeptidase serum levels were raised in 21.9%, 61% and 53.7% of patients, respectively.

Five patients underwent right hepatectomy (12.2%), nine left hepatectomy (22%), twenty six bisegmentectomy 2-3 (63.4%) and one patient underwent a segment 5 resection. A Roux-en-Y hepaticojejunostomy was associated with liver resection in 14 patients as follows: seven with bilateral and seven (21.8%) with unilateral disease and common bile duct dilation larger than 2 cm in diameter. Another three patients with unilateral stones who had previously been submitted to hepaticojejunostomy were submitted to liver resection and the anastomosis was maintained. All patients had a drain placed at the site of resection.

There was no operative mortality. Two patients submitted to liver resection (right hepatectomy and bisetorectomy 2-3) in a septic condition had an uneventful outcome. Four patients (9.8%) had a postoperative biliary fistula and were conservatively managed with an uneventful outcome; one patient (2.4%) developed a right subphrenic abscess which was percutaneously drained with good outcome.

Thirty two patients with unilateral and two with bilateral disease (82.9%) had good long-term results. Seven patients (17.1%), 2 with unilateral and 5 with bilateral stones, had late complications of the disease: cholangitis associated with recurrent stones in three (bilateral disease); cholangitis in two (unilateral disease); liver abscess associated with recurrent stones in one and liver abscess in one (all with bilateral stones).

One of these patients had caudate lobe recurrent stones and an abscess 93 mo after resection of segments 2 and 3, and died 28 d after drainage of the abscess due to sepsis; one had a liver abscess percutaneously drained with good outcome; three patients with cholangitis and stone recurrence, received antibiotic therapy and percutaneous stone removal and have remained well; two patients with cholangitis were treated with systemic antibiotics with good outcome. The long-term mortality rate was 2.4%.

The overall rate of good long-term results was 82.9% and was 94.1% and 28.6%, respectively for unilateral and bilateral disease. Comparing the data of good results between patients with unilateral and bilateral disease, statistical analysis showed a significant difference ($P < 0.001$) (Table 1).

All patients submitted to liver resection only, showed good long-term results (100%), while seven of seventeen patients (41.2%) who underwent liver resection associated with hepaticojejunostomy had late postoperative complications. A comparison between liver resection alone and resection associated with hepaticojejunostomy showed a statistically significant difference ($P = 0.0006$) (Table 1).

Twenty seven out of 34 patients with unilateral disease were submitted to liver resection alone and all had a good outcome. Of the remaining seven patients with unilateral disease who were submitted to liver resection associated with a bilioenteric anastomosis, two had recurrence of symptoms (2/7, 28.5%). A comparison between liver resection alone and resection associated with hepaticojejunostomy for patients with unilateral disease, showed a statistically significant difference ($P = 0.0498$).

DISCUSSION

Primary intrahepatic lithiasis is a rare disease in Western countries but, the high number of cases diagnosed in our institution, led to a treatment protocol based on presentation of the disease[2,9], where 41 out of 98 patients with symptomatic hepatolithiasis underwent liver resection.

The aim of treatment was the removal of intrahepatic and extrahepatic stones as well as duct strictures and to promote adequate drainage of the remaining segments of the biliary tree. Liver resection is the only treatment that can achieve these goals, thus reducing the risk of recurrence[4,5,7,8,10-14]. In this series, liver resection was indicated in patients with irreversible lesions such as biliary strictures or severe parenchymal fibrosis or atrophy, criteria initially proposed by Choi and Wong[6] and employed by many others[4,7,15,16].

Hepatic resection for the treatment of hepatolithiasis can lead to low rates of cholangitis or stone recurrence and good long-term results ranging from 80% to 98%[3-5,7,10-13,16]. In this series, good late results were observed in 100% of the patients submitted to liver resection only, showing that in some situations cure of the disease is possible.

With regard to the long-term results, seven patients (17.1%), 2 with unilateral and 5 with bilateral stones, all submitted to liver resection and bilioenteric anastomosis, had complications: five had cholangitis and two had liver abscesses. One of these patients died and the other 6 were treated successfully.

Patients with unilateral disease had significantly better results compared to those with bilateral stones, 94.1% and 28.6% had good late results, respectively. These data are comparable to other reports from the Far East and to our own previous experience, where good results were achieved in 80% to 100% of patients with unilateral stones and in 50% to 80% of those with bilateral disease[3-7,9-11]. These results can be explained by the fact that in patients with unilateral disease, all the compromised liver parenchyma is removed, potentially leading to cure of the disease, while the same is not always possible in those with bilateral disease. Indeed, if one looks at our data, good late results were achieved in all patients with unilateral stones who did not present with extrahepatic biliary disease. However, if stones were present in the remnant parenchyma or there was a dilation of the extrahepatic biliary tree and a biliary drainage procedure and hepaticojejunostomy was required, the rate of good results fell significantly to 58.8%. This was probably due to two factors: (1) Associated extrahepatic biliary disease (persistence of a possible cause for stone formation and/or inadequate biliary or stone drainage); and (2) Bilateral disease (persistence of affected liver tissue).

Most authors emphasize that at long-term follow-up, patients submitted to liver resection associated with a bilioenteric anastomosis, have a worse prognosis when compared to those submitted to resection only[4,8]. In recent years, reports have shown higher rates of postoperative cholangitis in patients submitted to hepaticojejunostomy[17-19].

Although patients submitted to hepaticojejunostomy had a higher incidence of poor late results, it is difficult to state whether cholangitis in these cases was due to recurrent stones in the remnant liver or to the presence of a bilioenteric anastomosis. Indeed, Roux-en-Y hepaticojejunostomy is the procedure of choice because the long jejunal loop is employed to avoid bacterial reflux into the liver. In an attempt to solve this question, we compared only patients with unilateral disease, with and without hepaticojejunostomy and, despite a small number of patients; there was a significant difference between the groups

showing a direct effect of the bilioenteric anastomosis on patient outcome.

Although the majority of groups perform a Roux-en-Y hepaticojejunostomy in patients with bilateral stones, the real benefits of this procedure have not yet been proven. Indeed, Li *et al*[19] showed that stones located in the lateral and posterior segments of the liver, do not drain easily through the biliary anastomosis. Moreover, Chen *et al*[20] showed excellent results employing percutaneous treatment without any surgical treatment in patients with bilateral stones. According to this data and reinforced by the poor results in our patients with hepaticojejunostomy, a biliary anastomosis may not be the ideal solution for these patients. Further studies are needed to establish the best treatment for bilateral hepatolithiasis and for those with unilateral disease and a dilated extrahepatic duct.

This study with the largest non-oriental series of primary intrahepatic lithiasis showed that liver resection can lead to the cure of unilateral hepatolithiasis. However, in patients with bilateral disease and in those with extrahepatic biliary duct dilation, where a hepaticojejunostomy was performed, more than 30% of patients had symptom recurrence and a rigorous follow-up is necessary. For the late group of patients, other treatment modalities such as resection associated with percutaneous treatment instead of hepaticojejunostomy should be considered.

COMMENTS

Background

Surgical treatment of primary intrahepatic lithiasis in a Western country was evaluated. The paper reports the largest non-oriental series of liver resection for hepatolithiasis. Prognostic factors were evaluated and bilateral disease treated with a bilioenteric anastomosis had a negative impact on outcome.

Research frontiers

It may be necessary for surgeons who deal with this challenging disease to reevaluate the benefit of hepaticojejunostomy.

Innovations and breakthroughs

Evaluation of prognostic factors in patients submitted to surgical treatment of primary intrahepatic lithiasis.

Applications

The benefit of other treatment modalities such as resection associated with percutaneous treatment instead of hepaticojejunostomy in patients with extrahepatic biliary duct dilation and for those with bilateral intrahepatic stones.

Peer review

This is an interesting manuscript on a challenging group of patients.

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BRIEF ARTICLE

Prevalence of type 2 diabetes in Algerian patients with hepatitis C virus infection

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Abstract

AIM: To investigate the prevalence of, and risk factors for, diabetes mellitus (DM) in Algerian patients with chronic hepatitis C virus (HCV) infection and in a control group.

METHODS: A cross-sectional study was undertaken. A total of 416 consecutive patients with viral chronic hepatitis attending the Internal Medicine Department of the University Hospital Center Touhami Benflis in Batna [290 HCV-infected and 126 hepatitis B virus (HBV)-infected patients] were prospectively recruited.

RESULTS: The prevalence of DM was higher in HCV-infected patients in comparison with HBV-infected patients (39.1% *vs* 5%, $P < 0.0001$). Among patients without cirrhosis, diabetes was more prevalent in HCV-infected patients than in HBV-infected patients (33.5% *vs* 4.3%, $P < 0.0001$). Among patients with cirrhosis, diabetes was more prevalent in HCV-infected patients, but the difference was not significant (67.4% *vs* 20%, $P = 0.058$). The logistic regression analysis showed that HCV infection [odds ratio (OR) 4.73, 95% CI: 1.7-13.2], metabolic syndrome (OR 12.35, 95% CI: 6.18-24.67), family history of diabetes (OR 3.2, 95% CI: 1.67-6.13) and increased hepatic enzymes (OR 2.22, 95% CI: 1.1-4.5) were independently related to DM in these patients.

CONCLUSION: The high prevalence of diabetes in HCV-infected patients, and its occurrence at early stages of hepatic disease, suggest that screening for glucose abnormalities should be indicated in these patients.

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Key words: Prevalence; Hepatitis C virus; Hepatitis B virus; Diabetes mellitus; Algeria

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INTRODUCTION

Hepatitis C virus (HCV) infection is a common worldwide medical problem; it is one of the major causes of chronic liver disease. According to recent World Health Organization estimates the worldwide prevalence of HCV infection is 2.2%, affecting approximately 130 million people globally[1]. Patients with chronic HCV infection may develop various extrahepatic manifestations, including cryoglobulinemia, the presence of serum antibodies, glomerulonephritis, sialoadenitis and porphyria cutaneous tarda[2]. Several studies from different parts of the world have reported that HCV infection may also contribute to the development of diabetes mellitus (DM), and higher prevalence of type 2 DM has been observed in patients with HCV infection than in those with other forms of chronic hepatitis[3-5]. However, the prevalence of type 2 DM in patients with HCV infection has not been reported in Algeria, as far as we know.

Thus, in order to examine the prevalence of DM in patients with chronic HCV infection in Batna (Algeria), we conducted a cross-sectional study assessing the prevalence of this metabolic disorder in patients with HCV infection in comparison with the prevalence in those with hepatitis B virus (HBV) infection. In addition, risk factors associated with DM development such as age, body mass index (BMI), diabetic familial history and metabolic syndrome were also evaluated to clarify the possible role of chronic HCV infection in association with development of diabetes.

In this study, the prevalence of diabetes in patients with chronic HCV has not been compared to that in the general population, because patients with chronic liver disease, regardless of etiology, have a higher prevalence of diabetes mellitus[6]. We have chosen patients with chronic hepatitis B as a control group, because HBV infection is the second leading cause of chronic hepatitis after HCV in Algeria[7].

MATERIALS AND METHODS

Patients

From September 2004 to September 2007, we conducted a cross-sectional study by enrolling patients with chronic viral hepatitis admitted to the University Hospital Center Touhami Benflis in Batna, Algeria. The diagnosis of HCV infection was made if patients were positive for anti-HCV antibody and HCV RNA. The presence of anti-HCV antibody was assessed using the third generation microparticle enzyme immunoassay test. The presence of HCV RNA was confirmed by Cobas Ampliprep/Roche Taq Man (Pasteur Institute, Algiers and Sadelaoud Laboratory, Batna, Algeria). HBV infection was diagnosed if patients had evidence of hepatitis B surface antigen. Patients with concomitant HCV and HBV infection were excluded. There was no serologic evidence of co-infection with other hepatotropic viruses or with human immunodeficiency virus. Patients having other causes of liver disease, in particular those known to be involved in the pathogenesis of diabetes, such as hemochromatosis or alcoholic liver disease, were excluded. None of the study patients had received corticosteroids during the previous 6 mo before the study. Patients with a history of, or evidence of, pancreatitis, pancreatic tumor, hepatic tumor or cirrhosis with Child-Pugh category C were excluded from the study. None of the study patients had previously received anti-viral treatment. No woman was pregnant in this study. Patients who were infected with HCV or HBV after being diagnosed with diabetes were also excluded from the analysis.

According to the American Diabetes Association criteria[8], patients were assigned a diagnosis of DM if they were using oral hypoglycemic medication or insulin, or if they showed fasting glucose greater than 126 mg/dL on two occasions, or glucose greater than 200 mg/dL, 2 h after an oral glucose tolerance test, performed in patients with impaired fasting glucose (fasting glucose concentration $\geqslant$ 110 mg/dL and $<$ 126 mg/dL).

A diagnosis of cirrhosis was established either by histology or by presumptive diagnosis made when patients had ascites, hematologic evidence of hypersplenism, esophageal varices or relevant ultrasonographic findings. Liver biopsy was performed in patients with increased alanine aminotransferase (ALT) and who gave their informed consent beforehand. Liver biopsy specimens were analyzed by a single experienced pathologist, who was informed of clinical and biologic data. Fibrosis was assessed using the METAVIR score[9]. Fibrosis stage (F) was scored as F0 (absent), F1 (portal fibrosis), F2 (portal fibrosis with few septa), F3 (septal fibrosis), and F4 (cirrhosis).

The BMI and family history of DM were recorded for each patient during enrollment. The BMI was expressed as the body weight divided by the square of the body length (kg/m^2). Overweight was defined as a BMI 25-29.9 kg/m^2 and obesity as a BMI $\geqslant$ 30 kg/m^2. The family history of diabetes was obtained from the patients themselves and was recorded as positive if their first-degree relatives had DM. The metabolic syndrome was diagnosed according to the National Cholesterol Education Program's Adult Treatment Panel Ⅲ definition[10].

Statistical analysis

All data values were expressed as mean ± standard deviation. Results were compared between HCV and HBV patients using the χ^2 test for categorical variables and Student *t*-test for continuous variables. Stepwise multivariate logistic regression was performed to evaluate the predictive variables associated with the presence of diabetes in the study patients. All statistical analyses were performed using Epi info 2000 (Statistics Program for Public Health. CDC, Atlanta, USA), and a P value < 0.05 was considered significant.

RESULTS

In total, 416 patients (290 HCV-infected patients and 126 HBV-infected patients) were enrolled in this study. Considerable differences could be noted when the demographic characteristics of the two groups were compared (Table 1).

Table 1 Characteristics of all patients in the chronic viral hepatitis cohort *n* (%)

Clinical features	Virological diagnosis		*P*
	HBV (*n* = 126)	HCV (*n* = 290)	
Age (yr)			
< 40	57 (45.2)	13 (4.5)	< 0.001
40-59	60 (47.6)	193 (66.6)	< 0.001
⩾ 60	9 (7.1)	84 (29)	< 0.001
Male sex	79 (62.7)	78 (26.9)	< 0.001
Family history of diabetes	38 (30.2)	103 (35.5)	0.28
BMI (kg/m^2), mean ± SD	25.22 ± 4.48	26.27 ± 4.51	0.05
BMI ⩾ 25 kg/m^2	68 (54)	161 (55.5)	0.42
Metabolic syndrome	20 (15.9)	122 (42.1)	< 0.0001
Increased ALT	15 (11.9)	177 (61)	< 0.0001
Cirrhosis	5 (4)	51 (17.6)	0.00018

HBV: Hepatitis B virus; HCV: Hepatitis C virus; BMI: Body mass index; ALT: Alanine aminotransferase.

Table 2 Analysis of variables associated with diabetes in chronic viral hepatitis *n* (%)

Variables	Virological diagnosis		*P*
	HBV (*n* = 121)	HCV (*n* = 261)	
Diabetic	6 (5)	102 (39.1)	< 0.0001
Age (yr)			
< 40	0/56 (0)	1/12 (8.3)	
40-59	4/58 (6.9)	61/174 (35.1)	< 0.0001
⩾ 60	2/7 (28.6)	40/75 (52.6)	0.56
Family history of diabetes	1/37 (2.7)	52/92 (56.5)	< 0.0001
BMI ⩾ 25 kg/m^2	5/66 (7.6)	70/147 (47.6)	< 0.0001
Metabolic syndrome	4/18 (22.2)	75/108 (69.4)	< 0.001
Increased ALT	2/14 (14.3)	79/155 (51)	0.01
Cirrhosis	1/5 (20)	29/43 (67.4)	0.058
No cirrhosis	5/116 (4.3)	73/218 (33.5)	< 0.0001

HBV: Hepatitis B virus; HCV: Hepatitis C virus; BMI: Body mass index; ALT: Alanine aminotransferase.

Patients with hepatitis C were older than those with hepatitis B (55 ± 9 years *vs* 40 ± 13 years, $P < 0.0001$). Percentage of men was lower in the HCV-infected patients than in those with HBV (26.9% *vs* 62.7%, $P < 0.0001$). In addition, there were significantly more patients with metabolic syndrome among the hepatitis C patients, and more cirrhosis in the hepatitis C group.

DM was observed more often in HCV-infected patients than in HBV-infected patients (39.1% *vs* 5%, $P < 0.0001$). However, this difference is statistically significant only in patients aged between 40 and 60 years.

We compared variables associated with diabetes in patients with patent diabetes (6 HBV-infected patients and 102 infected HCV patients) and in those who were non-diabetics (115 HBV-infected patients and 159 HCV-infected patients). Patients with impaired fasting glucose (5 HBV-infected patients and 29 HCV-infected patients) were excluded from this comparison.

A family history of diabetes appeared to be matched in the present study; of the subjects infected with HCV and HBV, 35.5% and 30.2%, respectively, had a familial history of DM. Patients with a family history of diabetes were more likely to have DM compared with those without (41.08% *vs* 21.73%, $P < 0.0001$). For patients with a family history of DM, the prevalence of diabetes was significantly higher in subjects with HCV infection compared with those with HBV infection (56.5% *vs* 2.7%, $P < 0.000001$) (Table 2).

Metabolic syndrome was more frequent in HCV-infected patients (42.1% *vs* 15.9%, $P < 0.0001$). Patients with metabolic syndrome were more likely to have DM compared with those without (62.69% *vs* 11.32%, $P < 0.000001$). For patients with metabolic syndrome, DM was significantly more frequent in HCV-infected patients than in those with HBV infection (69.4% *vs* 22.2%, $P < 0.001$). In obese or overweight subjects (BMI ⩾ 25 kg/m^2), DM was more frequent in HCV-infected patients (47.6% *vs* 7.6%, $P < 0.0001$).

Liver disease appeared more severe in the HCV group. Cirrhosis was more frequent in HCV-infected patients

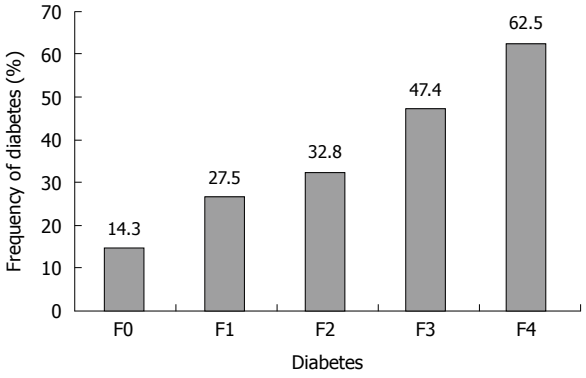

Figure 1 Frequency of diabetes by stage of fibrosis in hepatitis C virus-infected patients.

than in those with HBV infection (17.6% *vs* 4%, $P < 0.001$). In patients without cirrhosis, DM was more frequent in patients with HCV infection than in those with HBV infection (33.5% *vs* 4.3%, $P < 0.00001$). However, in patients with cirrhosis, diabetes was more prevalent in HCV-infected patients, but the difference was not significant (67.4% *vs* 20%, $P = 0.058$).

In HCV-infected patients in whom liver biopsy was performed, DM prevalence increased progressively and significantly with the fibrosis stage (Figure 1). DM was more frequent in patients with increased ALT plasma concentration (> 40 IU/L) compared with those with normal ALT plasma concentration (47.92% *vs* 12.67%, $P < 0.00001$). In patients with increased ALT plasma concentration, DM was significantly more frequent in HCV-infected patients than in those with HBV infection (51% *vs* 14.3%, $P = 0.01$).

The multiple regression analysis revealed that the major independent variables associated with type 2 diabetes were metabolic syndrome [odds ratio (OR) 12.35, $P = 0.0001$, 95% CI: 6.18-24.67], HCV infection (OR 4.73, $P = 0.0029$, 95% CI: 1.69-13.20), family history of diabetes (OR 3.2, $P = 0.0004$, 95% CI: 1.67-6.13) and increased ALT (OR 2.22, $P = 0.027$, 95% CI: 1.09-4.52) (Table 3).

Table 3 Factors associated with the development of diabetes in patients chronically infected with hepatitis virus

Variables	Odds ratio	95% CI	*P* value
Metabolic syndrome	12.35	6.18-24.67	0.00001
Hepatitis C	4.73	1.69-13.20	0.0029
Family history of diabetes	3.2	1.67-6.13	0.0004
Increased ALT	2.22	1.09-4.52	0.027

ALT: Alanine aminotransferase.

DISCUSSION

Our epidemiological and virological data suggest that HCV infection is more closely related to diabetes than HBV infection. Diabetes was observed in 39.1% of patients with HCV infection, as compared with 5% of HBV-infected subjects in our population. However, our study has some limitations, related to the small size of the control group. Indeed, chronic hepatitis B is much less common than chronic hepatitis C in our area[7]. Our findings are in concordance with similar epidemiological studies from different part of the world.

Allison *et al*[11] published, in 1994, the first article about a link between viral hepatitis C and diabetes. In their retrospective study of 100 cirrhotic patients listed for transplantation, these authors reported that the prevalence of type 2 DM was higher in patients with HCV-associated cirrhosis than in cirrhotics with other underlying liver diseases. In a cross-sectional survey including 9841 persons, Mehta *et al*[12] found that HCV-positive persons who were older than 40 years had an increased risk for type 2 diabetes mellitus, more than 3-fold when compared to persons without HCV infection. However, no difference was seen between HBV-infected subjects and the general population[12].

In a retrospective analysis of 1117 patients with chronic viral hepatitis, diabetes was present in significantly more patients with HCV compared to those with HBV infection (21% *vs* 12%)[13]. In a separate case-control trial included in the same report, the prevalence of HCV infection was significantly higher among patients with diabetes than among controls (4.2% *vs* 1.6%).

Diabetes mellitus has been more often seen in cirrhotic patients[14]. However, in a cohort of 45 non-cirrhotic patients with chronic hepatitis C the prevalence of type 2 DM was 33%, higher than in the matched control group and in a group of patients with chronic hepatitis B[15]. Furthermore, in a large retrospective study DM was present in 23.6% of patients with hepatitis C, and in 9.4% of those with hepatitis B[16].

Recently, in a Spanish study which included 525 chronic hepatitis C patients treated with peginterferon plus ribavirin, patients were followed up after treatment. The incidence of altered baseline glucose and the appearance of type 2 DM was greater in non-responders than in sustained responders, even after multivariate analysis including such confounding variables as previous type 2 DM in relatives, age older than 40 years and male sex. Thus, hepatitis C virus clearance induced a decrease in insulin resistance index during short time follow-up and decreased the incidence of type 2 DM in long-term follow-up[17].

Shintani *et al*[18], in an experimental model, observed that the HCV core antigen transgenic mouse had higher basal insulin levels than non-transgenic mice, and readily developed diabetes when fed a high-fat diet, in addition to exhibiting marked insulin resistance as demonstrated by the insulin tolerance test.

In the present study, logistic regression analysis confirmed that family history of diabetes, metabolic syndrome and increased transaminases were the major independent variables associated with DM. This finding is consistent with reports in the literature[19-21].

The mechanisms by which hepatitis C induces increased insulin resistance and the risk for development of diabetes has not been completely understood. Liver fibrosis progression has long been considered responsible for the appearance of insulin resistance and type 2 DM in patients with chronic liver diseases[22]. However, in our study, diabetes occurs in the early stages of liver disease. The mechanism through which HCV is associated with insulin resistance involves direct viral effects, proinflammatory cytokines and suppressors of cytokine signaling[23-25].

In conclusion, this study shows a higher prevalence of DM in patients with HCV infection than in those with HBV infection, and that DM occurs at an early stage of hepatic disease. However, other factors such as metabolic syndrome, family history of diabetes and increased transaminases seem also to be important risk factors for the development of diabetes in Algeria.

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COMMENTS

Background

A higher prevalence of diabetes mellitus (DM) has been observed in patients with hepatitis C virus (HCV) infection than in those with other forms of chronic hepatitis and several mechanisms have been implicated in the pathogenesis of DM. However, there is no information from Algeria regarding this issue, and few reports from Africa.

Research frontiers

Recent data link HCV infection with diabetes. However, diabetes is a multifactorial disease; other factors such as age, weight, family history of diabetes and cirrhosis contribute to the development of diabetes.

Innovations and breakthroughs

This is a cross-sectional study assessing the prevalence of diabetes in patients with HCV infection in comparison with the prevalence in those with hepatitis B virus (HBV) infection. It is the first study of its kind performed in Algeria. In addition, risk factors associated with DM development were also evaluated.

Applications

Diabetes plays a role in the initiation and progression of liver injury. The high prevalence of diabetes in HCV-infected patients, and its occurrence at early stages of hepatic disease, suggest that screening for glucose abnormalities should be indicated in these patients.

Peer review

The authors used 290 HCV-infected and 126 HBV-infected patients. It would be better if they had used equal numbers for both the groups or slightly fewer.

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BRIEF ARTICLE

Association of *E-cadherin* (*CDH1*) gene polymorphisms and gastric cancer risk

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Abstract

AIM: To investigate the associations between *CDH1* gene polymorphisms and gastric cancer (GC) risk predisposition.

METHODS: We analyzed four *CDH1* polymorphisms (*+54 T>C*, *-160 C>A*, *-616 G>C*, *-3159 T>C*) in an Omani population, by extraction of genomic DNA from the peripheral blood of 192 patients with GC and 170 control participants and performed *CDH1* genotyping using DNA sequencing.

RESULTS: *CDH1 -160 -AA* genotype was associated with an increased risk of GC (OR = 3.6, 95% CI: 1.1-11.8) (P = 0.03). There was no significant association between the other polymorphisms and GC risk. The haplotype analysis of *+54 T>C*, *-160 C>A*, *-616 G>C*, *-3159 T>C* genotypes revealed that the OR of *CCGC* and *CAGC* haplotypes was 1.5 (95% CI: 0.7-3.5) and 1.5 (95% CI: 0.2-3.0), but did not reach statistical significance.

CONCLUSION: The current study suggests that the *-160 AA* genotype was associated with an increased risk of GC in Oman.

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Key words: Gastric cancer; Polymorphism; *CDH1*

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INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and second most common cause of cancer mortality worldwide; therefore, it remains a global health burden[1,2]. GC has been associated with *Helicobacter* infection and environmental factors such as smoking, salted fish, and low intake of fruit and vegetables[3,4]. However, while these factors might affect large proportions of some populations, only subsets of these populations develop GC, and

therefore, increased genetic susceptibility has been postulated. Possible genetic risk factors have included single nucleotide polymorphisms (SNPs) in several pathways involved in chronic inflammation of gastric mucosa and subsequent carcinogenesis. The involved SNPs affect agents such as pro-inflammatory cytokines, xenobiotic metabolizing enzymes, and growth factors[5-11]. The study of these molecular pathways has helped to identify individuals at higher risk, particularly when examined with *Helicobacter pylori* (*H. pylori*) infection and other environmental exposure[7,8].

Adhesion molecules, especially the calcium-dependent intercellular adhesion molecule E-cadherin and its *CDH1* gene (located on chromosome 16), play a central role in carcinogenesis and metastasis[10,12]. The *CDH1* gene encodes a transmembrane glycoprotein that mediates intercellular adhesion and cellular polarity. The E-cadherin protein is a tumor invasion suppressor, and loss of its function results in transition to an invasive phenotype in human epithelial cancers[10,12].

Several SNPs in the *CDH1* gene are associated with GC. The most widely studied polymorphism is *CDH1 -160C>A*, where the *A* allele decreases transcriptional activity of the *CDH1* gene and E-cadherin expression, and increases susceptibility to GC in some populations[9,13-19]. Moreover, several other SNPs, including *+54 T>C*, *-3159 T>C*, *-160 C>A*, *-2076 C>T* and *-616 G>C*, were studied in Japanese and Italian populations, which resulted in the identification of haplotypes associated with increased risk of GC[12,20].

The above studies have highlighted the ethnic variation in frequency and risk predisposition of these SNPs[15,16]. Therefore, we studied in an Omani population, four *CDH1* gene polymorphisms (*+54 T>C*, *-160 C>A*, *-616 G>C* and *-3159 T>C*) that were previously examined in Japanese and Italian populations[12,20]. We evaluated the potential association of these SNPs and their haplotypes with GC susceptibility in a case-control design.

MATERIALS AND METHODS

Study participants

The study population consisted of a series of unrelated patients with GC who were diagnosed at two main hospitals in the Sultanate of Oman (Sultan Qaboos University Hospital and Royal Hospital). The healthy control group comprised persons of the same ethnic and geographical origin as the patients. The Medical Research and Ethics Committee of the College of Medicine of Sultan Qaboos University approved the study design. The study participants provided informed consent prior to participation, in compliance with the Declaration of Helsinki.

Genotyping method

From each participant, 10 mL blood was collected in an EDTA tube and stored frozen until the extraction of the DNA. DNA was extracted from whole blood using a commercial DNA blood kit (Gentra Puregene DNA Purification kit; Qiagen, Gaithersburg, MD, USA) and stored until processing for genotyping.

Analysis of the *CDH1* SNPs, *+54 T>C*, *-160 C>A*, *-616 G>C* and *-3159 T>C*, was performed using multiplex polymerase chain reaction (PCR) with an ABI premix. Genomic DNA from whole blood was used as a PCR template in a total reaction volume of 10 μL that contained 10 pmol designed primers: *+54 T>C (rs3743674)*: [5'-CCCCTGGTCTCATCATTTC-3' (forward) and 5'-AATTCCTCCAAGAATCCCCAG-3' (reverse)]; *160 C>A (rs16260)*: [5'-TGATCCCAGGTCTTAGTGAG-3' (forward) and 5'-GCTCCTCAGGACCCGAAC-3' (reverse)]; *-616 G>C (rs7203904)*: [5'-TTGACTGAGGCCACAGAGTG-3' (forward) and 5'-CTGCCTAAATCTGCTGAGCC-3' (reverse)]; *-3159 T>C* (*rs2010724*): [5'-GAGCTTCCCAGAGCCTTTCT-3' (forward) and 5'-ATTGGACTTGCCAAGGGTG-3' (reverse)]. PCR was performed as follows: one cycle at 94℃ for 10 min, 35 cycles at 94℃ for 30 s, 59℃ for 30 s, and 72℃ for 30 s, followed by 72℃ for 5 min. The final extension was at 72℃ for 10 min. PCR products were analyzed on a 2.5% agarose gel stained with ethidium bromide and photographed under UV light. The PCR product was subsequently sequenced in an ABI PRISM 3100 sequencer using BigDye Terminator v3.1 Cycle Sequencing method (Applied Biosystems,USA) as recommended by the manufacturer. Candidate SNP regions were detected and typed with the aid of DNA Star Software (DNASTAR, Madison, WI, USA).

Statistical analysis

The genotypic distributions of different polymorphic loci in the control samples were compared with those expected from the Hardy-Weinberg equilibrium using the χ^2 test. The differences in frequency distributions of the genotypes between the patient and control groups were also tested using the χ^2 test. Age- and sex-adjusted ORs and 95% CIs were calculated using logistic regression analysis. Haplotype frequencies, haplotype-survival analyses, and standardized disequilibrium coefficients (D) were calculated using Thesias software available at http://genecanvas.ecgene.net/. $P < 0.05$ was considered statistically significant. Analysis of data was performed using SPSS version 10.0 software (SPSS, Chicago, IL, USA).

RESULTS

One hundred and ninety-two GC patients and 170 unrelated controls were included. The age range for the participants included in the study was 19-80 years, and the mean ages for the patients and controls were 55.1 ± 12.5 and 32.8 ± 6.6 years, respectively. The percentages of male and female participants were 58.3% and 41.7% for GC patients respectively, and 56.5% and 43.5% for controls. *H. pylori* infection status was available in 116 GC patients and 90 control participants, with a positivity rate of 58% and 60% (Table 1). Most GC patients in this cohort presented at an advanced stage, with slight predominance of non-intestinal type according to Lauren's classification, as shown in Table 2.

Table 1 Demographic data, *Helicobacter* status, and smoking in gastric cancer patients and control subjects

Variable	GC patients	Control
No. of subjects	192	170
Age (yr), mean ± SD	32.8 ± 6.6	55.1 ± 12.5
Male, %	58.30	56.50
H. pylori status, *n*	116[1]	90[1]
Positive, *n* (%)	67 (58)	54 (60)

[1]The number of GC patients and control participants for whom *Helicobacter pylori* (*H. pylori*) serology was available. GC: Gastric cancer.

Table 2 Clinicopathological features of 192 gastric cancer patients

Variable	*n* (%)
Lauren's classification	
Intestinal	93 (48.5)
Mixed and diffuse	99 (51.5)
Histological grade	
G1	11 (5.8)
G2	80 (41.6)
G3	101 (52.6)
T stage	
T1 + T2	32 (16.7)
T3 + T4	160 (83.3)
Lymph node involvement	
Negative	26 (13.5)
Positive	166 (86.5)
TNM stage	
Ⅰ + Ⅱ	33 (17.2)
Ⅲ + Ⅳ	159 (82.8)

CDH1 genotypic frequencies and GC risk

The frequencies of the *+54 T>C*, *-160 C>A*, *-616 G>C* and *-3159 T>C* genotypes are shown in Table 3. The SNP analysis was successful in the majority of GC patients and control subjects, however, 15-23 samples failed for GC patients and 4-13 samples for control subjects, as shown in Table 3. The allelic distributions for control subjects did not deviate significantly from those expected from the Hardy-Weinberg equilibrium. There was a significant association between the *CDH1-160 AA* genotype, with an increased risk of GC, with OR 3.6 (95% CI: 1.1-11.8, $P = 0.03$) (Table 3). There was no significant association between the other *CDH1* polymorphisms and GC risk (Table 3).

Table 3 *CDH1* genotype frequencies and their associated risk of gastric cancer predisposition

CDH1 genotype	Patients *n* (%)[1]	Control *n* (%)[1]	OR[2] (95% CI)	*P* value
+54 T>C	*n* = 174	*n* = 157		
TT	25 (14.4)	22 (14.0)	1	
TC	70 (40.2)	75 (47.8)	0.9 (0.4-2.2)	0.9
CC	79 (45.4)	60 (38.2)	0.9 (0.4-2.4)	0.8
CC + TC	149 (85.6)	135 (86.0)	0.9 (0.4-2.1)	0.8
TT + TC	95 (54.6)	97 (61.8)	1.0 (0.5-2.0)	1.0
C allele	66.0	62.0		
-160 C>A	*n* = 174	*n* = 166		
CC	93 (53.6)	93 (56.0)	1	
CA	60 (34.5)	65 (39.2)	0.6 (0.3-1.1)	0.1
AA	21 (12.0)	8 (4.8)	3.6 (1.1-11.8)	0.03
AA + CA	81 (46.5)	73 (44.0)	0.8 (0.4-1.5)	0.8
CC + CA	153 (88.1)	158 (95.2)	3.4 (1.4-13.9)	0.01
A allele	29.0	24.0		
-616 G>C	*n* = 172	*n* = 159		
GG	84 (48.8)	71 (44.7)	1	
GC	65 (37.8)	69 (43.4)	0.9 (0.6-1.8)	0.7
CC	23 (13.4)	19 (12.0)	1.8 (0.6-5.1)	0.3
GC + CC	88 (51.2)	88 (55.4)	1.0 (0.6-1.9)	0.9
GG + GC	149 (86.6)	140 (88.1)	1.8 (0.7-5.2)	0.3
C allele	32.0	34.0		
-3159 T>C	*n* = 177	*n* = 166		
TT	52 (29.7)	47 (28.3)	1	
TC	72 (41.1)	78 (47.0)	0.9 (0.4-1.7)	0.7
CC	53 (30.3)	41 (24.7)	1.0 (0.5-2.0)	0.9
CC + TC	125 (71.4)	119 (71.7)	0.9 (0.5-1.7)	0.8
TT + TC	124 (70.8)	125 (75.3)	1.1 (0.54-2.2)	0.8
C allele	50.0	48.0		

[1]The number of patients and control indicates successful single nucleotide polymorphism analysis for each polymorphism; [2]Age and sex-adjusted.

Haplotype analysis

The common haplotypes were identified, as shown in Table 4. There were significant differences in the distribution of these haplotypes between patients and controls (Table 4). The haplotype analysis of *+54 T>C*, *-160 C>A*, *-616 G>C* and *-3159 T>C* genotypes revealed that the OR of *CCGC* and *CAGC* haplotypes was 1.5 (95% CI: 0.7-3.5) and 1.5 (95% CI: 0.2-3.0), respectively, but did not reach statistical significance.

Table 4 Frequencies of *CDH1* haplotypes and associated risk of gastric cancer predisposition

Haplotype	Frequency (%)		OR[1] (95% CI)	*P* value
	Patient	Control		
TCGT	20.1	22.1	1	
TACG	10.4	11.0	0.99 (0.5-1.9)	1.0
CCGT	16.5	17.7	1.0 (0.5-1.8)	1.0
CCGC	7.0	5.0	1.5 (0.7-3.5)	0.3
CCCT	11.1	9.9	1.1 (0.5-2.3)	0.8
CCCC	15.1	16.6	0.9 (0.6-1.6)	0.8
CAGC	10.7	7.1	1.5 (0.8-3.0)	0.2
CACC	5.8	5.7	1.1 (0.5-2.4)	0.8

[1]Age and sex-adjusted.

DISCUSSION

Six polymorphisms of the *CDH1* gene have been studied previously in Caucasian, East Asian, and Mexican populations and included: *-616 G>C*, *-160 C>A*, *-3159 T>C*, *+54 T>C*, *2076C>T* and *347G>GA*[12-17,20]. A recent meta-analysis has highlighted the role of ethnic differences by showing that the associations between these polymorphisms and GC among Asian and Caucasian populations are in opposite directions[15,18]. Therefore, we investigated the association between GC and the *CDH1 +54 T>C*, *-160 C>A*, *-616 G>C* and *-3159 T>C* polymorphisms in an Omani population, an ethnic group in which the association between GC and these polymorphisms has not been studied previously.

The most widely studied *CDH1* polymorphism in various cancers is *CDH1 -160 C>A*[13-19]. In the present study, we found that this polymorphism affected the risk of developing GC. The carriage of the *CDH1 -160 AA* genotype increased the risk of GC (OR: 3.6, 95% CI: 1.1-11.8) (P = 0.03). Two meta-analyses have suggested that the association of *CDH1 -160 AA* with GC risk is ethnicity-dependent, whereby the OR estimates for *CDH1 -160 AA* carriers are less than 1.0 for Asians but significantly greater than 1.0 for Caucasians[15,18]. Thus, our results are consistent with the findings in Caucasian populations. The explanation for this observation remains unclear, however, the A variant decreases transcription efficiency by 68% compared with the C allele *in vitro*[9]. The altered expression of adhesion molecule E-cadherin results in tumor development and carcinogenesis. Possible explanations for the discrepancy between ethnic groups include the frequency of the polymorphism in the population studied or linkage disequilibrium with other, perhaps undiscovered, functional SNPs in the *CDH1* gene. The present study shows that there is no association between the *CDH1 +54 T>C* and *-616 G>C* SNPs and GC development. Although a study by Zhang *et al*[13] has found an association between *+54 T>C* and esophageal and gastric cancer, other studies were negative[15].

It has been suggested that haplotype analysis might be more useful than single SNP analysis in identifying cancer risk[12,20]. In particular, the combined analysis of *CDH1 -160 C>A*, *-2076C>T* and *+54 T>C* has suggested that a haplotype ATT increases susceptibility to GC, whereas the CTT haplotype has a protective effect[12,20]. Yamada *et al* have studied the *+54 T>C*, *-160 C>A*, *-616 G>C*, *-2076 T>C* and *3159 T>C* polymorphisms and have found that the TCGTT haplotype is the most common haplotype and has a protective effect, whereas the TAGTC haplotype increases susceptibility to GC[12,20]. The haplotype analysis of *+54 T>C*, *-160 C>A*, *-616 G>C* and *-3159 T>C* genotypes revealed that the OR of *CCGC* and *CAGC* haplotypes was 1.5 (95% CI: 0.7-3.5) and 1.5 (95% CI: 0.2-3.0), respectively, but did not reach statistical significance. The reason for the difference can be attributed to differences in polymorphisms studied, genetic background and local environmental factors, and highlights the need for comparative studies between different ethnic groups.

In conclusion, the current study confirms the ethnic variations in the association between *CDH1 -160 C>A* polymorphisms and GC susceptibility. We demonstrated that the *-160 AA* genotype was associated with an increased risk of GC. This finding could allow the identification of higher-risk groups who might benefit from intensive prevention strategies (aimed at infections or environmental factors). A better understanding of the functional aspects of these polymorphisms in tumor tissue could lead to a better understanding of tumor biology and behavior, and elucidate the discrepancies observed between and within studies.

COMMENTS

Background

E-cadherin plays a central role in carcinogenesis and metastasis. *E-cadherin* (*CDH1*) gene polymorphisms at various loci and their significance for predisposition to gastric cancer (GC) risk have been studied previously with different results that have suggested ethnic variation. The authors investigated the associations between *CDH1* gene polymorphisms and GC risk predisposition.

Research frontiers

A better understanding of *CDH1* gene polymorphisms in GC could lead to a better understanding of tumor biology and behavior.

Innovations and breakthroughs

The current study confirms the ethnic variations in the association between *CDH1 -160 C>A* polymorphisms and GC susceptibility. The authors demonstrated that the *-160 AA* genotype was associated with an increased risk of GC.

Applications

These findings could allow the identification of higher-risk groups who might benefit from intensive prevention strategies (aimed at infections or environment factors).

Terminology

CDH1 gene encodes E-cadherin protein, which is an important adhesion molecule. Single nucleotide polymorphisms are DNA sequence variations that occur when a single nucleotide is altered.

Peer review

This study provides some useful epidemiological information about genetic predisposition and the risk of GC.

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BRIEF ARTICLE

Transcatheter arterial chemoembolization with a fine-powder formulation of cisplatin for hepatocellular carcinoma

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Abstract

AIM: To evaluate the efficacy of transcatheter arterial chemoembolization (TACE) using a suspension of a fine-powder formulation of cisplatin (DDPH) for hepatocellular carcinoma (HCC).

METHODS: The study population was comprised of 164 patients who were treated by TACE alone. Of these patients, 76 underwent TACE using a suspension of DDPH in lipiodol (LPD) (DDPH group), and the remaining 88 underwent TACE with an emulsion of doxorubicin (ADM) with LPD (ADM group). We compared the DDPH group with the ADM group in terms of the objective early response rate, progression free survival (PFS) and overall survival (OS).

RESULTS: The objective early response rate in the DDPH group was significantly higher than that in the ADM group (54% *vs* 24%, $P < 0.001$). The PFS rate in the DDPH group was also significantly higher than that in the ADM group ($P < 0.001$). Moreover, the OS in the DDPH group was significantly longer than that in the ADM group ($P = 0.002$). Although the incidence rate of nausea or vomiting in the DDPH group was higher than that in the ADM group, the ADM group showed a higher incidence rate of the adverse events of hepatic arterial damage and leucopenia. No other serious complications were observed in either group.

CONCLUSION: We conclude that TACE using a suspension of DDPH in LPD could be a useful treatment for HCC.

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Key words: Hepatocellular carcinoma; DDPH; Transcatheter arterial chemoembolization; Cisplatin; Doxorubicin

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Kasai K, Ushio A, Sawara K, Miyamoto Y, Kasai Y, Oikawa K, Kuroda H, Takikawa Y, Suzuki K. Transcatheter arterial chemoembolization with a fine-powder formulation of cisplatin for hepatocellular carcinoma. *World J Gastroenterol* 2010; 16(27): 3437-3444 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3437.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3437

INTRODUCTION

Hepatocellular carcinoma (HCC) is the cancer with the sixth highest incidence in the world[1]. The number of deaths from HCC is also increasing throughout the world[2-5]. Development of new treatments for HCC has

helped improve the patient prognosis[6,7]. Local ablating therapies such as percutaneous ethanol injection (PEI) or radiofrequency ablation (RFA) have been effective in cases of limited tumor spread and are increasingly used[7,8]. However, the majority of patients are not eligible for these modalities because of large tumor size or diffuse tumor growth. In these patients regional transcatheter arterial chemoembolization (TACE) has been widely used as a palliative treatment[9,10]. Two randomized trials from Europe and Asia recently confirmed a survival benefit after TACE using gelfoam and iodized oil (lipiodol) compared to conservative treatment[11,12]. In recent years, TACE using an emulsion of doxorubicin (ADM) with lipiodol (LPD) (ADM-LPD emulsion) followed by embolization with a gelatin sponge has been employed commonly for HCC treatment[13,14]. However, the tumors have been demonstrated to show a high frequency of recurrence after TACE[10,15,16]. Cisplatin (CDDP), a platinum compound, is an effective anticancer agent used in the treatment of various malignancies[17]. Researchers have recently reported that TACE using a suspension of CDDP powder in LPD may be more effective against unresectable HCC as compared with TACE using ADM-LPD emulsion[18,19]. However, only limited institutions have used this for TACE because it is laborious to refine the CDDP powder. Since 2004, a fine-powder formulation of CDDP (DDPH, IA-call; Nippon Kayaku, Tokyo, Japan) has also been available as a therapeutic agent for intra-arterial infusion in Japan. As a result, TACE using DDPH has become widespread in Japanese institutions. Nevertheless, the efficacy of TACE using DDPH-LPD suspension has not yet been reported.

In this article, we compared the effectiveness with regard to the response rate (RR), progression free survival (PFS) and overall survival (OS) between TACE using a suspension of DDPH in LPD (DDPH-LPD suspension) and ADM-LPD emulsion. Moreover, we analyzed the prognostic factors for clinical outcome of patients treated with TACE.

MATERIALS AND METHODS

Patients

Between January 2006 and July 2009, 164 HCC patients who showed no indication for surgical resection or local ablation therapy such as RFA and PEI therapy were enrolled in the study. HCC was diagnosed by the distinctive findings on ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI) and angiography, and the serum levels of des-γ-carboxy prothrombin (DCP) and α-fetoprotein (AFP). Histologic examination was not always carried out. Liver function was evaluated according to the Child-Pugh classification[20]. Tumor stage was judged by the TNM classification established by the International Union Against Cancer[21]. The extent of portal vein invasion was classified as follows: Vp 0, no invasion of the portal vein; Vp 1, invasion of the third or more distal branch of the left or right portal vein; Vp 2, invasion of the second branch of the portal vein; Vp3, invasion of the first branch of the portal vein; and Vp4, invasion of the trunk of the portal vein. After being presented with the clinical results of previous studies of TACE using DDPH-LPD suspension or TACE using ADM-LPD emulsion, all 164 patients themselves selected the therapeutic option on the basis of informed consent. All of the enrolled patients met the eligibility criteria for inclusion in the analysis described in the next paragraph. The patients were divided into two groups: one group consisting of 76 patients who underwent TACE using DDPH-LPD suspension (DDPH group), and another group consisting of 88 patients who underwent TACE using ADM-LPD emulsion (ADM group). They were all treated by TACE alone.

Informed consent was obtained from all of the patients. The study protocol was approved by the Ethics Committee of Iwate Medical University and the study was conducted in accordance with the Declaration of Helsinki 1975.

Eligibility criteria

The eligibility criteria of the patients for this study were as follows: (1) No indication for surgical resection or local ablation therapy such as RFA and PEI therapy; (2) No evidence of extra-hepatic metastasis; (3) No tumor thrombus in the main trunk of portal vein; (4) No evidence of active heart or renal diseases meeting the contraindications for ADM and CDDP therapy, respectively; (5) Eastern Cooperative Oncology Group (ECOG) performance status (PS)[22] level 0-2; (6) Hypervascular tumors showing enhancement during angiography; (7) Bidimensionally measurable hepatic lesions; (8) No uncontrolled ascites or pleural effusion; and (9) Total serum bilirubin (T-Bil) less than 3 mg/dL.

The presence of underlying liver diseases such as hepatitis or cirrhosis was confirmed by laboratory, radiological examinations and pathological examinations. We classified the chronic hepatitis patients into Child-Pugh class A, because chronic hepatitis is a known precirrhotic condition.

Preparation of the agents for TACE

We used DDPH or ADM (Adriacin; Kyowa Hakko Kogyo, Tokyo, Japan) mixed with LPD (iodized oil; Andre Guerget, Aulnay-sous-Bois, France).

The DDPH-LPD suspension was prepared by mixing 50 mg of DDPH into 3-10 mL of LPD.

The ADM-LPD emulsion was prepared by the following procedure: 10-30 mg of ADM was dissolved in 1-2 mL of a contrast medium (Iomeron; Eisai Co., Ltd., Tokyo, Japan) and then mixed with 3-10 mL LPD.

The dosage of LPD and the anticancer drugs was adjusted depending on the tumor size, number of tumors, degree of liver impairment and renal function, however, the maximum dose of LPD was not allowed to exceed 10 mL.

Treatments

Hepatic arteriography, superior mesenteric arterial porto-

venography, CT during arteriography and CT during arterio-portography were performed to define the size and locations of tumor nodules and to exclude tumor thrombus in the main trunk of the portal vein. Following hepatic angiography, a catheter was selectively inserted into the hepatic artery supplying the target tumor and the DDPH-LPD suspension or the ADM-LPD emulsion was injected. In patients with several tumors in the liver, superselective catheterization was performed for each lesion. If superselective catheterization was not possible, the DDPH-LPD suspension or the ADM-LPD emulsion was injected into the right and left main hepatic artery distal to the origin of the cystic artery. After the injection, arterioembolization was performed used gelatin sponge particles (Gelpart; Nippon Kayaku, Tokyo, Japan) mixed with contrast medium.

All the patients were followed up with US, CT and/or MRI after 1 mo and every 3 mo thereafter. TACE was undertaken again when relapse of the treated lesions and/or new hepatic lesions were detected. These patients received additional TACE using the same agent during the follow-up period. The TACE was repeated until complete regression of the tumor was obtained, or until the patient could no longer be treated.

Post treatment assessment

Early tumor response was assessed by US, CT and/or MRI, conducted 1 mo after the initial treatment. We regarded LPD accumulation in the tumor as representing a necrotic area, based on previous reports of such LPD retention areas corresponding to the necrotic areas on CT[23-26]. By measurement of the two largest perpendicular diameters of the tumor, we classified the tumor response into four categories using the following criteria: complete response (CR), complete disappearance or 100% necrosis of all tumors; partial response (PR), reduction and/or necrosis, with at least 50% decrease of all the measurable lesions; progressive disease (PD), an increase of the tumor size exceeding 25% of all the measurable lesions or appearance of a new lesion; stable disease (SD), disease not qualifying for classification as CR, PR, PD.

Toxicity was evaluated by the National Cancer Institute-Common Terminology Criteria for Adverse Events, version 3.0 (CTCAE v3.0).

Statistical analysis

The differences in the background clinical characteristics of the patients between the DDPH group and ADM group were assessed by Mann-Whitney's *U* test, logistic regression test, or the χ^2 test, as appropriate.

PFS and OS were calculated from the date of start of the therapy to the date on which tumor progression was documented and the date of death of the patient, respectively. Both were assessed by the Kaplan-Meier life-table method, and the differences between the two treatment groups were evaluated by the log rank test. Univariate analysis to identify the predictors of survival in the patients was conducted by the Kaplan-Meier life-table method, and the differences between the two groups were evaluated by the log rank test. Multivariate analysis to identify the predictors of survival was conducted using the Cox proportional hazards model. Statistical significance was defined as a *P* value of less than 0.05. All of the above analyses were performed using the SPSS software (version 11, SPSS, Chicago, IL, USA).

Table 1 Patient characteristics

Characteristics	DDPH group	ADM group	*P* value
No. of patients	76	88	
Age (yr) [median, (range)]	67 (32-87)	69 (21-90)	0.093
Gender (male/female)	57/19	52/36	0.031
Etiology (HBV/HCV/NBNC)	11/50/15	8/64/16	0.508
Child-Pugh classification (A/B/C)	47/26/3	45/36/7	0.303
TNM classification (Ⅰ-Ⅱ/Ⅲ-Ⅳ)	10/66	24/64	0.026
Tumor size (≤ 3.0/> 3.0 cm)	21/55	30/58	0.373
Number of tumors (1-3/≥ 4)	35/41	46/42	0.427
PVTT (Vp0-2 /Vp3)	62/14	80/8	0.080
Total bilirubin (≤ 1.5/> 1.5 mg/dL)	66/10	75/13	0.906
Albumin (≤ 3.5/> 3.5 g/dL)	38/38	45/42	0.822
AFP (≤ 1000/> 1000 ng/mL)	68/8	79/8	0.776
DCP (≤ 1000/> 1000 mAU/mL)	59/14	73/14	0.609

Data are expressed as median with range values, or the number of patients. The stages of HCC by TNM classification are clustered into two groups (Ⅰ-Ⅱ and Ⅲ-Ⅳ). The tumor characteristics and other parameters are classified as follows: tumor size: ≤ 3.0, > 3.0 cm; tumor number: 1-3, > 4; extent of PVTT: Vp 0-2, and Vp 3; serum bilirubin: ≤ 1.5, > 1.5 mg/dL; serum albumin: ≤ 3.5, > 3.5 g/dL; serum AFP levels: ≤ 1000, > 1000 ng/mL. Serum DCP levels: ≤ 1000, > 1000 mAU/mL. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NBNC: Negative for hepatitis B surface antigen and HCV antibody; PVTT: Portal vein tumor thrombosis; AFP: α-fetoprotein; DCP: Des-γ-carboxy prothrombin; DDPH group: Using a suspension of DDPH in lipiodol (LPD); ADM group: With an emulsion of doxorubicin (ADM) with LPD.

RESULTS

Patient profile

The characteristics of the 164 patients of both groups are summarized in Table 1. There were 109 male and 55 female patients, ranging in age from 21 to 90 years old (mean, 68 years old).

Regarding the assessment of differences in the characteristics of the patients, there were significant differences in the gender distribution and in the TNM classification between the two groups, i.e. there was a higher proportion of males ($P = 0.031$) and more subjects with advanced TNM classification ($P = 0.026$) in the DPHH group. There were no significant differences in any of the other characteristics between the two groups.

Treatments and early tumor response

The median follow-up period was 13.1 mo (range: 1-40 mo). We performed 392 TACE procedures (157 sessions in the DDPH group, 235 sessions in the ADM group) in 164 patients. The median number of TACE

Table 2 Early tumor response *n* (%)

	DDPH (*n* = 76)	ADM (*n* = 88)	*P* value
CR	2 (3)	5 (6)	
PR	39 (51)	16 (18)	
SD	23 (30)	5 (6)	
PD	12 (16)	62 (70)	
CR + PR	41 (54)	21 (24)	< 0.001

Data are expressed as number of patients and percentages. DDPH group: Using a suspension of DDPH in lipiodol (LPD); ADM group: With an emulsion of doxorubicin (ADM) with LPD; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

Table 3 Univariate analysis for identifying the predictors of survival

Variable	Hazard ratio	95% CI	*P* value
Treatment regimen (ADM *vs* DDPH)	0.580	0.325-1.035	0.065
Age (≤ 65 yr *vs* > 65 yr)	1.286	0.741-2.231	0.372
Gender (female *vs* male)	1.651	0.944-2.888	0.079
Etiology (NBNC *vs* HBV/HCV)	0.734	0.432-1.246	0.252
Child Pugh classification (A *vs* B/C)	1.142	0.689-1.891	0.607
TNM classification (Ⅰ-Ⅱ *vs* Ⅲ-Ⅳ)	2.765	1.252-6.106	0.012
Tumor size (≤ 3.0 cm *vs* > 3.0 cm)	2.094	1.161-3.776	0.014
Number of tumors (1-3 *vs* ≥ 4)	2.612	1.535-4.444	0.001
PVTT (Vp0-2 *vs* Vp3)	4.714	2.520-8.819	< 0.001
Total bilirubin (≤ 1.5 mg/dL *vs* > 1.5 mg/dL)	1.730	0.874-3.422	0.116
Albumin (≤ 3.5 g/dL *vs* > 3.5 g/dL)	0.996	0.603-1.647	0.989
AFP (≤ 1000 ng/mL *vs* > 1000 ng/mL)	1.323	0.528-3.315	0.551
DCP (≤ 1000 mAU/mL *vs* > 1000 mAU/mL)	2.396	1.288-4.459	0.005

DDPH group: Using a suspension of DDPH in lipiodol (LPD); ADM group: With an emulsion of doxorubicin (ADM) with LPD; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NBNC: Negative for hepatitis B surface antigen and HCV antibody; PVTT: Portal vein tumor thrombosis; AFP: α-fetoprotein; DCP: Des-γ-carboxy prothrombin.

procedures was 2 sessions (range: 1-5 sessions) in the DDPH group and 3 sessions (range: 1-6 sessions) in the ADM group. The median interval to the re-treatment with TACE was 9.4 mo in the DDPH group and 3.8 mo in the ADM group. One hundred and ten sessions (70.1%) in the DDPH group and 170 sessions (72.3%) in the ADM group were treated by superselectivity of TACE. There was no significant difference in the incidence of superselectivity of TACE between the two groups.

In the DDPH group, 2 (3%), 39 (51%), 23 (30%) and 12 (16%) patients showed CR, PR, SD and PD, respectively. In the ADM group, 5 (6%), 16 (18%), 5 (6%) and 62 (70%) patients showed CR, PR, SD and PD, respectively. Therefore, the objective early response rate of the DDPH group (54%) was significantly higher than that in the ADM group (24%). The difference in the rate between the two groups was statistically significant ($P < 0.001$) (Table 2).

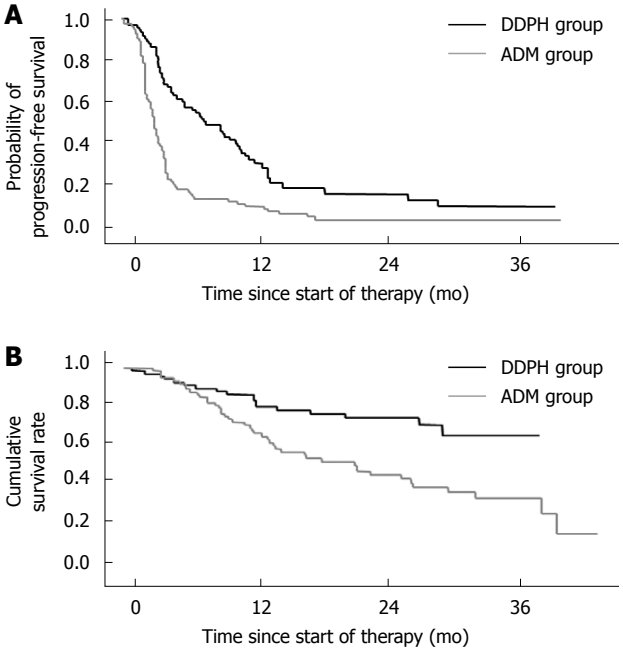

Figure 1 Comparison of the progression-free survival rates (A) and overall survival (B) between the DDPH and ADM groups. A: The progression-free survival rate was significantly higher in the DDPH group than in the ADM group (log-rank test: $P < 0.001$); B: The overall survival was significantly longer in the DDPH group than in the ADM group (log-rank test: $P = 0.002$). DDPH group: Using a suspension of DDPH in lipiodol (LPD); ADM group: With an emulsion of doxorubicin (ADM) with LPD.

PFS

The median PFS was 8.6 mo in the DDPH group and 3.0 mo in the ADM group. The PFS rates at 6, 12, 24 and 36 mo were 58%, 32%, 18% and 11%, respectively, in the DDPH group. In contrast, the corresponding values were 18%, 10%, 5% and 5%, respectively, in the ADM group. The PFS rates in the DDPH group were significantly higher than those in the ADM group ($P < 0.001$) (Figure 1A).

Survival

The median survival time (MST) in the DDPH and ADM groups was "not reached" and 20.8 mo, respectively. The OS values at 6, 12, 24, and 36 mo were 92%, 81%, 76% and 67%, respectively, in the DDPH group. The corresponding values in the ADM group were 87%, 68%, 46% and 37%, respectively. The OS in the DDPH group was significantly longer than that in the ADM group ($P = 0.002$) (Figure 1B).

Univariate analysis to identify the predictors of survival indicated five possible factors affecting the survival: TNM classification; tumor size; number of tumors; portal vein tumor thrombosis (PVTT) and serum DCP level. The treatment regimen was close to being statistically significant ($P = 0.065$) for survival (Table 3). Multivariate analysis performed using factors that were considered

Table 4 Multivariate analysis for identifying predictors of survival

Variable	Hazard ratio	95% CI	*P* value
Treatment regimen (ADM *vs* DDPH)	0.329	0.149-0.726	0.006
Gender (female *vs* male)	2.291	1.174-4.470	0.015
Number of tumors (1-3 *vs* ≥ 4)	6.541	3.201-13.363	< 0.001
PVTT (Vp0-2 *vs* Vp3)	6.704	2.581-17.418	< 0.001
Albumin (≤ 3.5 g/dL *vs* > 3.5 g/dL)	0.311	0.157-0.612	0.001

DDPH group: Using a suspension of DDPH in lipiodol (LPD); ADM group: With an emulsion of doxorubicin (ADM) with LPD; PVTT: Portal vein tumor thrombosis.

significant ($P < 0.1$) on univariate analysis identified the treatment regimen, gender, number of tumors, PVTT, and serum albumin as independent factors affecting the survival (Table 4).

Adverse effects

Table 5 shows a summary of the adverse effects in the two groups. The incidence rate of nausea/vomiting in the DDPH group was significantly higher than that in the ADM group ($P < 0.001$). In addition, the incidence rates of hepatic arterial damage (HAD) after TACE and leucopenia in the ADM group were significantly higher than those in the DDPH group ($P < 0.001$ and $P = 0.002$, respectively). We observed HAD in 17 patients. Although one patient in the DDPH group was observed to have slight wall irregularity of the hepatic artery (HA), HAD associated with TACE did not interfere with catheterization at the next TACE session. On the other hand, in the ADM group, we observed slight wall irregularity of HA in six patients, overt stenosis of HA in four patients and occlusion of HA in six patients. In six patients who were observed as having occlusion of HA, it became impossible to treat with repeated TACE.

No other serious complications or treatment-related deaths were observed in either group.

Table 5 Adverse events *n* (%)

Adverse effect	Treatment group (%)		*P* value
	DDPH group (*n* = 76)	ADM group (*n* = 88)	
Nausea/vomiting	64 (84)	48 (55)	< 0.001
Fever	61 (80)	73 (83)	0.571
Abdominal pain	53 (69)	63 (72)	0.958
Elevation of transaminase levels	55 (72)	62 (71)	0.993
Liver abscess	1 (1)	2 (2)	0.765
Hepatic arterial damage	1 (1)	16 (18)	< 0.001
Renal or liver failure	0 (0)	2 (2)	0.229
Leucopenia	3 (4)	12 (14)	0.002
Thrombocytopenia	4 (5)	6 (7)	0.650
Fatigue	21(28)	27 (31)	0.839

DDPH group: Using a suspension of DDPH in lipiodol (LPD); ADM group: With an emulsion of doxorubicin (ADM) with LPD; Data are expressed as number of patients, with the percentages indicated in parentheses.

DISCUSSION

TACE has been widely used for the treatment of unresectable HCC[9,10]. The most commonly used agent used in TACE for HCC treatment is ADM-LPD emulsion, followed by embolization with a gelatin sponge[13,14]; however, the tumors frequently recur[10,15,16] or residual tumors are observed at a high incidence. CDDP is an effective anticancer agent used in the treatment of various malignancies[17]. It has been reported to exert its actions by binding to the DNA in cancer cells, inhibiting DNA synthesis and subsequent cellular division. The antitumor activity of CDDP is closely associated with the serum concentration of the drug[27]. Therefore, the antitumor activity can be enhanced by increase of the dose. LPD acts as a selective carrier of anticancer agents and as an embolic material[23]; the anticancer agent is gradually released from the iodized oil. Although the mechanism of topical accumulation of LPD in the tumor is not yet precisely understood, it is used nonetheless to achieve a targeting drug delivery system with long-lasting accumulation in the tumor and gradual drug release. Consequently, augmented antitumor efficacy and milder side-effects have come to be expected with the use of this substance for TACE. In fact, Morimoto *et al*[28] investigated the pharmacological advantages of TACE using DDPH for hypervascular hepatic tumors in animal experiments. They reported that the tumor concentration of the platinum agent in the DDPH-LPD-TACE group was about 14 times higher than that in the DDPH-hepatic arterial infusion (HAI) group. In addition, they reported that the plasma concentrations of the platinum agent at 5 and 10 min from start of the infusion were lower in the DDPH-LPD-TACE group than those in the DDPH-HAI group. Recently, Ono *et al*[18] reported that TACE using a suspension of CDDP powder in LPD was more effective than that using ADM-LPD emulsion against unresectable HCC. Other investigators have also frequently reported favorable results obtained with TACE using a suspension of CDDP powder in LPD in HCC patients[19,29]. However, the CDDP powder for this therapy is difficult to produce because of the characteristics of the drug formulation. Therefore, CDDP powder had to be a custom-made formulation in individual institutions[30]. Consequently, when an institution was able to dispense CDDP powder in its own pharmacy department, TACE using a suspension of CDDP powder in LPD was undertaken.

A fine-powder formulation of CDDP, namely "DDPH", for intra-arterial infusion has been available for HCC treatment since 2004 in Japan. Dispensing of CDDP powder improved with the development of DDPH, and DDPH has now come to replace CDDP powder. Using DDPH-LPD suspension for TACE in HCC patients was expected to yield better therapeutic outcomes; therefore, TACE using DDPH became widespread in Japanese institutions. Nevertheless, the efficacy of TACE using DDPH-

LPD suspension has not yet been reported. Therefore, we compared the outcomes of TACE using DDPH-LPD suspension and ADM-LPD emulsion.

Analysis of the results in our study revealed that the objective response rate in the DDPH group was significantly higher than that in the ADM group. Moreover, the OS of the patients in the DDPH group was significantly longer than that of the patients in the ADM group. This could be explained as being due to the fact that TACE with ADM cannot be repeated as required because of the high frequency of adverse effects of ADM such as leucopenia, severe vascular changes and occlusion of the hepatic artery[18,31,32]. In fact, the incidences of leucopenia and HAD in the ADM group in our study were significantly higher than those in the DDPH group. Considering the fact that TACE is often repeated in most patients, longer patency of the hepatic artery is preferable for properly deploying the lipiodol mixture and embolic agents into the tumor. In addition, we conclude that anthracyclines such as ADM may be relatively less effective against HCC; this is because of the high expression level of P-glycoprotein, which transports antitumor agents such as anthracyclines or vinca alkaloids from cells with a high active efflux mechanism, in HCC tumors[33].

On the other hand, Pelletier *et al*[34] reported that TACE with CDDP sometimes caused severe complications, such as acute hepatic failure. The treatment also did not produce any significant improvement of the survival rate in this study. Severe complications could be expected with the high doses (2 mg/kg) of CDDP used in their study. Therefore, we performed TACE using DDPH-LPD suspension in our study with half of the dose (50 mg = 1 mg/kg) that they had used. Modification of the CDDP dose used for the treatment to DDPH 50 mg in our study resulted in a lower severity of complications.

Takayasu *et al*[35] reported a nationwide prospective cohort study which was performed in 8510 patients with unresectable HCC who underwent TACE using an emulsion of lipiodol and anticancer agents followed by gelatin sponge particles as an initial treatment. In their report, multivariate analysis for the factors affecting survival showed significant differences in degree of liver damage, AFP, maximum tumor size, number of lesions, and PVTT. In contrast to their report, we could not observe AFP value as a prognostic factor in our multivariate analysis. This may be due to fewer in the study population and a shorter observation period in our study compared with their study. In addition, a cut-off value for AFP of 1000 ng/mL in our study was much higher than that (400 ng/mL) in their study because we aimed to analyze the difference in the effect of TACE with the extent that HCC had progressed. Therefore, we could not observe AFP value as a prognostic factor in our multivariate analysis.

This study was not a well-controlled prospective study. Nevertheless, the patients in the two groups had fairly similar characteristics with regard to age, etiology, Child-Pugh classification, tumor size, number of tumors, PVTT, total bilirubin, albumin, AFP, and DCP. In relation to the differences in the characteristics of the patients, the DDPH group had a significantly higher proportion of males and a more advanced stage in TNM classification than the ADM group. Several investigators[36,37] have shown that TNM classification and tumor stage are independent prognostic factors for survival of patients who are treated by TACE. Therefore, we forecast that the prognosis of the CDDP group was worse than that of the ADM group, because the DDPH group had more advanced stage in TNM classification than the ADM group. However, the OS in the DDPH group was significantly longer than that in the ADM group. Moreover, to avoid the confounding effects of any deviations in the patient characteristics causing an impact on the results, we used the multivariate analysis for comparison of the efficacy between the regimens. The analysis identified the treatment regimen employed for the TACE as one of the most important prognostic factors. Compared to a previous report[18] describing TACE using a suspension of CDDP powder in LPD, the objective response rate and OS in the DDPH group in our study were significantly higher.

Considering these facts, we conclude that TACE using DDPH-LPD suspension could be a useful treatment strategy for HCC patients. To confirm these results, randomized controlled trials comparing TACE using DDPH-LPD suspension with TACE using ADM-LPD suspension for patients with HCC are mandatory.

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COMMENTS

Background

In recent years, transcatheter arterial chemoembolization (TACE) using an emulsion of doxorubicin (ADM) with lipiodol (LPD) (ADM-LPD emulsion) followed by embolization with a gelatin sponge has been employed commonly for hepatocellular carcinoma (HCC) treatment. However, the tumors have been demonstrated to show a high frequency of recurrence after TACE.

Research frontiers

Cisplatin, a platinum compound, is an effective anticancer agent used in the treatment of various malignancies. Since 2004, a fine-powder formulation of cisplatin (DDPH, IA-call; Nippon Kayaku, Tokyo, Japan) has also been available as a therapeutic agent for intra-arterial infusion in Japan. Researchers have recently reported that TACE using a suspension of cisplatin powder in LPD may be more effective against unresectable HCC as compared with TACE using ADM-LPD emulsion. Therefore, TACE using DDPH has become widespread in Japanese institutions. However, the efficacy of TACE using DDPH-LPD suspension has not yet been reported.

Innovations and breakthroughs

In this article, the authors reported the effectiveness of TACE using DDPH-LPD suspension compared with that using ADM-LPD emulsion.

Applications

Although randomized controlled trials comparing TACE using DDPH-LPD suspension with TACE using ADM-LPD suspension for patients with HCC are needed, this study shows that TACE using DDPH-LPD suspension can be a useful treatment strategy for HCC patients.

Terminology

TACE: Transarterial chemoembolization, a procedure in which the blood supply to a tumor is blocked (embolized) and chemotherapy is administered directly into the tumor.

Peer review

Kasai *et al* evaluated the efficacy of TACE using a suspension of DDPH for HCC. The authors indicated that early response rate, progression free survival and overall survival in the DDPH group was significantly higher than that in the ADM group.

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BRIEF ARTICLE

Association of genetic polymorphisms of aldehyde dehydrogenase-2 with esophageal squamous cell dysplasia

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Abstract

AIM: To demonstrate the possible associations between genetic polymorphisms of aldehyde dehydrogenase-2 (*ALDH2*) and esophageal squamous cell dysplasia (ESCD).

METHODS: All participants came from an area of high incidence of esophageal cancer and underwent an endoscopic staining examination; biopsies were taken from a non-staining area of the mucosa and diagnosed by histopathology. Based on the examinations, the subjects were divided into the control group with normal esophageal squamous epithelial cells and the ESCD group. *ALDH2* genotypes of 396 cases were determined including 184 ESCD cases and 212 controls. The odds ratio (OR) and 95% confidence intervals (95% CI) were calculated by binary logistic regression models.

RESULTS: The distribution of *ALDH2* genotypes showed significant differences between the two groups. The adjustment factors were gender and age in the logistic regression models. Compared with *2*2/2*2* genotype, *2*1/2*1* genotype was found to be a risk factor for ESCD, and the OR (95% CI) was 4.50 (2.21-9.19). There were significant correlations between *ALDH2* genotypes and alcohol drinking/smoking/history of esophageal cancer.

CONCLUSION: The *ALDH2* polymorphism is significantly associated with ESCD.

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Key words: Aldehyde dehydrogenase 2; Polymorphism; Alcohol; Smoking; Esophageal squamous cell dysplasia; History of esophageal cancer

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of

the most common malignant tumors, and alcohol consumption is a major risk factor for esophageal cancer[1-3]. Alcohol is first oxidized by alcohol dehydrogenase (ADH) to acetaldehyde[4,5], which is then oxidized to acetate by acetaldehyde dehydrogenase (ALDH). The *ALDH2* gene encoding ALDH2 is composed of 13 exons residing on chromosome 12. Deficiency at ALDH2 is a dominant trait that is caused by a single Glu to Lys amino acid substitution at residue 487, a change that can be attributed to a G-to-A transition (dbSNP: rs671) in exon 12 of the gene. This deficient allele (*ALDH2*487Lys*) is common in many East Asian populations[6,7], and approximately 45% of Chinese and Japanese individuals have inactive ALDH2 phenotype[8].

The *ALDH2* alleles encoding the active and inactive subunits are termed "*ALDH2*1*" and "*ALDH2*2*" respectively, and *ALDH2*2* exhibits functional polymorphism that is associated with a lower rate of alcohol dependence[6]. In persons with *ALDH2*2*, the body fails to metabolize acetaldehyde rapidly, leading to excessive accumulation of acetaldehyde, so *ALDH2*2* enhances the risk of esophageal squamous cell carcinoma in East Asian drinkers[9-11].

The stages of the carcinogenic process of esophageal cancer develop from normal esophageal mucosa to esophagitis, esophageal hyperplasia, dysplasia, and then to cancer in situ and early cancer[12]. It is also noteworthy that Japanese and Chinese pathologists prefer to classify "atypical" squamous epithelium dysplasia as a precancerous lesion. Historically, atypical squamous dysplasia has been classified as mild, moderate, or severe[13,14].

Most reports of case-control studies have only consisted of one case-group of esophageal cancer and one normal control group[15,16], and few reports based on population screening data found an association of *ALDH2* with esophageal squamous cell dysplasia (ESCD). Recently, we carried out such a screening survey for esophageal lesions in a high incidence area of esophageal cancer and performed sampling to determine the association of genetic polymorphism of *ALDH2* with the ESCD in this area.

MATERIALS AND METHODS

Study population

The subjects in this study consisted of 184 patients with ESCD and 212 controls with normal esophageal mucosa. All subjects in the present study were selected from the screened participants of Feicheng County between January 2004 and December 2006.

The screening included a cardiograph, ventral ultrasound, and endoscopic examination, which used mucosal stain with 1.2% iodine solution. The biopsies were taken from a non-stained area of mucosa, which then underwent pathologic evaluation carried out by two pathologists. Participating subjects who suffered from cardiovascular, liver and kidney diseases, cancers, and psychiatric disorders were excluded. A uniform questionnaire was used to interview all the subjects to obtain information such as socio-demographic characteristics, alcohol intake, tobacco use, and family history of esophageal carcinoma. The local ethics committee approved the study protocol, and all participants gave their written informed consent.

DNA extraction

A 3-5 mL elbow venous blood sample was collected at 9-10 am after a 12-h fast from each participant. The heparinized sample was centrifuged for 10 min at 3000 r/min to separate plasma and obtain blood cells. Genomic DNA was extracted using phenol-chloroform method and frozen at -20℃.

PCR amplification

The following two pairs of primers were produced by Takara Biotechnology (Dalian Co., Ltd.): F1, 5'-TCATGCCATGGCAACTCCAGC-3'; R1, 5'-CCCACACTCACAGTTTTCTCTTC-3'; F2, 5'-TACGGGCTGCAGGCATACACTA-3'; R2, 5'-TGATCCCCAGCAGGTCCTGAA-3'. F1 and R1 were used to amplify the *ALDH2*1* allele (296 bp), and F2 and R2 to amplify the *ALDH2*2* allele (203 bp). Two 25 microliters reaction tubes were needed for each specimen to amplify *ALDH2*1* (G) and *ALDH2*2* (A) respectively, each containing 30-100 ng DNA, 0.12 mmol/L dNTPs, 12.5 pmol F1 (or R1) primer, 12.5 pmol F2 (or R2) primer, 0.5 U *Taq* polymerase, and 2.5 μL 10 × PCR buffer (containing 15 mmol/L $MgCl_2$). The reaction tubes were heated to 95℃ for 5 min followed by 30 cycles of 95℃ for 60 s, 60℃ for 60 s, 72℃ for 60 s, and 72℃ for 45 s, and then followed by a final extension of 5 min at 72℃. Ten microliters PCR product was used in agarose gel electrophoresis and the electrophoresis result was photographed.

Electrophoresis results

Two lanes were used for each specimen. If one showed 296 bp band and the other showed no band, the corresponding genotype was *ALDH2*1/2*1* (G/G); if one showed 296 bp band and the other showed 203 bp band, the corresponding genotype was *ALDH2*1/2*2* (G/A); and if one showed 203 bp band and the other showed no band, the corresponding genotype was *ALDH2*2/2*2* (A/A).

Statistical analysis

Results for the enumeration of data (e.g. the number of individuals with various genotypes) and comparison of percentages between groups were evaluated with a χ^2 test. Allele frequencies were calculated using allele counting tests for the Hardy-Weinberg equilibrium by the chi-square, while the odd ratio (OR) and 95% confidence intervals (95% CI) were calculated by multinomial logistic regression model. The statistical analysis were made using SPSS program (version 11.5), and $P < 0.05$ (two-sided) was taken as statistically significant.

Table 1 Characteristics of cases and controls *n* (%)

Variables	Controls	Cases	t/χ^2	P^3
Age (yr)	51.01 ± 8.427	54.89 ± 8.443	-4.567	0.000
Income (yuan/yr per person)	1825 ± 1605	1768 ± 1599	0.350	0.726
Height (cm)	163.71 ± 7.374	164.40 ± 7.225	-0.944	0.346
Weight (kg)	67.08 ± 52.848	61.36 ± 8.027	0.671	0.502
Body mass index (kg/m^2)	23.08 ± 2.957	22.68 ± 2.462	1.470	0.142
SBP (mmHg)	132.45 ± 19.852	135.38 ± 20.411	-1.445	0.149
DBP (mmHg)	84.74 ± 13.083	85.27 ± 13.132	-0.406	0.685
Gender				
Male	123 (58.0)	124 (67.4)	3.687	0.055
Female	89 (42.0)	60 (184)		
Age (yr)				
40-49	94 (44.3)	41 (22.3)	22.407	0.000
50-59	81 (38.2)	91 (49.5)		
60-69	37 (17.5)	52 (28.3)		
Education				
Illiteracy	33 (15.6)	34 (18.5)	6.838	0.077
Primary school	54 (25.5)	54 (29.3)		
High school	96 (45.3)	85 (46.2)		
College and above	29 (13.7)	11 (6.0)		
Smoking index[1]				
0	117 (55.2)	81 (44.0)	6.107	0.047
< 500	47 (22.2)	43 (23.4)		
⩾ 500	48 (22.6)	60 (32.6)		
Alcohol drinking status[2]				
0	106 (50.0)	81 (44.0)	2.057	0.358
< 65	48 (22.6)	41 (22.3)		
⩾ 65	58 (27.4)	62 (33.7)		
History of esophageal cancer				
No	166 (84.7)	152 (82.6)	0.302	0.582
Yes	30 (15.3)	32 (17.4)		
ALDH2				
G/G	65 (30.7)	98 (53.3)	25.431	0.000
G/A	106 (50.0)	73 (39.7)		
A/A	41 (19.3)	13 (7.1)		

[1]Smoking index = cigarettes/d × number of smoking years; [2]Alcohol ⩾ 65 g/d = heavy drinker; [3]*t* test and χ^2 test were used for quantitative data variables and categorical data variables respectively. SBP: Systolic blood pressure; DBP: Diastolic blood pressure.

RESULTS

Basic data

All analysis variables are shown in Table 1. Gender, age, smoking, and *ALDH2* genotypes were significantly different between the two groups. The Hardy-Weinberg test for the control group showed the genotype distribution was in equilibrium. Gender and age as potential confounders were adjusted in the logistic models. Other variables including income (yuan/year per person), height, weight, body mass index, systolic blood pressure (SBP), diastolic blood pressure (DBP), alcohol drinking, and family history of esophageal cancer were not significantly different between the two groups.

Association of ALDH2 genotypes with ESCD

As shown in Table 2, after the potential confounders were adjusted, compared with *ALDH 2*2/2*2* genotype, *2*1/2*1* and *2*1/2*2* genotypes were related to having ESCD, and the ORs (95% CI) were 4.50 (2.21-9.19) and 2.19 (1.08-4.43), respectively. Meanwhile, after adjusting for gender and age, no significant association of smoking, alcohol drinking or family history of esophageal cancer with ESCD were observed in the two groups.

Table 2 Association of factors with squamous cell dysplasia of esophagus

Factors	OR (95% CI)[1]	OR (95% CI)[2]
Smoking index		
0	1.00	1.00
< 500	1.32 (0.80-2.18)	1.19 (0.64-2.24)
⩾ 500	1.81 (1.12-2.90)	1.56 (0.83-2.91)
Drinking index		
0	1.00	1.00
< 65	1.12 (0.67-1.86)	0.92 (0.49-1.76)
⩾ 65	1.40 (0.88-2.22)	1.03 (0.55-1.96)
History of esophageal cancer		
No	1.00	1.00
Yes	1.10 (0.66-1.85)	1.08 (0.64-1.83)
ALDH2		
A/A	1.00	1.00
A/G	2.17 (1.09-4.34)	2.19 (1.08-4.43)
G/G	4.76 (2.37-9.56)	4.50 (2.21-9.19)

[1]Crude OR; [2]Adjusted ORs were adjusted for age and gender. OR: Odds ratio; CI: Confidence intervals.

Interaction analysis of the ALDH2 genotypes and environmental factors

The frequency distribution of *ALDH2* genotypes combined with smoking index/alcohol drinking status and history of esophageal cancer are listed in Table 3.

As shown in Table 3, in the no smoking stratum only *ALDH 2*1/2*1* genotype increased the relative risk (OR = 6.97, 95% CI: 1.88-25.87); in the light smoking stratum *2*1/2*2* and *2*1/2*1* increased the relative risk and there was an interaction effect between the genotypes and smoking; and in the heavy smoking stratum either smoking or any genotypes of *ALDH2* were associated with the dysplasia, when compared with *ALDH2*2/2*2* genotype combined with no smoking as baseline.

In the no drinking stratum only *ALDH 2*1/2*1* genotype increased the relative risk (OR = 3.16, 95% CI: 1.19-8.41); and in the light and heavy drinking strata both *2*1/2*2* and *2*1/2*1* increased the relative risk with an interaction effect between the genotypes and drinking, when compared with *ALDH2*2/2*2* genotype combined with no drinking as baseline.

There was a significant interaction between *ALDH2*1/2*1* genotype and family history of esophageal cancer, when compared with *ALDH2*2/2*2* genotype and no family history of esophageal cancer as baseline.

DISCUSSION

Feicheng, a County in Shandong Province of China, was found to be a high incidence area of esophageal cancer. Its mortality rates from esophageal cancer were 63.19, 71.68, 66.87 and 82.33/100 000 in the years 1970-1974,

Table 3 Interaction between *ALDH2* genotypes and environmental factors for esophageal dysplasia *n* (%)

Factors	Genotype	Controls	Cases	OR (95% CI)[1]	OR (95% CI)[2]
Smoking index	*ALDH2*				
0	A/A	18 (8.5)	3 (1.6)	1.00	1.00
0	G/A	62 (29.2)	33 (17.9)	3.19 (0.88-11.64)	3.10 (0.84-11.45)
0	G/G	37 (17.5)	45 (24.5)	7.30 (1.99-26.71)	6.97 (1.88-25.87)
< 500	A/A	17 (8.0)	3 (1.6)	1.06 (0.19-5.99)	0.98 (0.17-5.77)
< 500	G/A	22 (10.4)	19 (10.3)	5.18 (1.32-20.35)	4.70 (1.16-19.07)
< 500	G/G	8 (3.8)	21 (11.4)	15.75 (3.63-68.41)	13.10 (2.85-62.14)
⩾ 500	A/A	6 (2.8)	7 (3.8)	7.00 (1.36-36.01)	6.84 (1.25-37.36)
⩾ 500	G/A	22 (10.4)	21 (11.4)	5.73 (1.47-22.33)	5.03 (1.21-20.82)
⩾ 500	G/G	20 (9.4)	32 (17.4)	9.60 (2.50-36.81)	7.78 (1.92-31.53)
Drinking index	*ALDH2*				
0	A/A	20 (9.4)	8 (4.3)	1.00	1.00
0	G/A	50 (23.6)	34 (18.5)	1.70 (0.67-4.30)	1.98 (0.75-5.25)
0	G/G	36 (17.0)	39 (21.2)	2.71 (1.06-6.91)	3.16 (1.19-8.41)
< 65	A/A	12 (5.7)	3 (1.6)	0.63 (0.14-2.82)	0.65 (0.19-3.09)
< 65	G/A	27 (12.7)	15 (8.2)	1.39 (0.48-3.91)	1.19 (0.40-3.50)
< 65	G/G	9 (4.2)	23 (12.5)	6.39 (2.07-19.69)	5.05 (1.57-16.15)
⩾ 65	A/A	9 (4.2)	2 (1.1)	0.56 (0.10-3.16)	0.43 (0.07-2.52)
⩾ 65	G/A	29 (13.7)	24 (13.0)	2.07 (0.78-5.23)	1.70 (0.60-4.80)
⩾ 65	G/G	20 (9.4)	36 (19.6)	4.50 (1.68-12.06)	3.19 (1.12-9.28)
History of esophageal cancer	*ALDH2*				
No	A/A	31 (15.8)	10 (5.4)	1.00	1.00
No	G/A	82 (41.8)	61 (33.2)	2.31 (1.05-5.06)	2.23 (1.01-4.93)
No	G/G	53 (27.0)	81 (44.0)	47.74 (2.15-10.47)	4.30 (1.93-9.62)
Yes	A/A	9 (4.6)	3 (1.6)	1.03 (0.23-4.58)	0.88 (0.20-4.01)
Yes	G/A	14 (7.1)	12 (6.5)	2.66 (0.93-7.59)	2.37 (0.82-6.89)
Yes	G/G	7 (3.6)	17 (9.2)	7.53 (2.42-23.37)	7.11 (2.25-22.45)

[1]Crude OR; [2]Adjusted ORs were adjusted for age and gender. OR: Odds ratio; CI: Confidence intervals.

1985-1989, 1990-1992, and 1997-1999, respectively[17]. In the present study, the cases and controls came from the same communities of Feicheng County, and they possessed similar environment and customs, so their data are comparable. All diseases were determined by endoscopic and pathological examinations, and the possibility of misclassification was small. Considering the information with little recall bias, we believe the results of this study provide more convincing evidence to elucidate the relationship between *ALDH2* polymorphism and ESCD.

The *ALDH2*2* allele produces an inactive protein subunit, which is unable to metabolize acetaldehyde. Genetic epidemiologic studies have suggested that the *ALDH2*2* allele inhibits the development of alcoholism. The inheritance of alcohol-induced flushing in families also suggested that the trait is dominant, that is, both *ALDH2*1/2*2* and *ALDH2*2/2*2* genotype encode inactive ALDH2[18,19].

The *ALDH2*1/2*1* allele has a dual effect on esophageal cancer. On one hand, it can convert acetaldehyde to acetate and get rid of the carcinogenic role of acetaldehyde. On the other, it decreases the blood level of acetaldehyde and alleviates adverse response to alcohol consumption[20], so individuals who have an *ALDH2*1/2*1* genotype are prone to heavy drinking and an increased risk of esophageal cancer. We found in this study that *ALDH2*1/2*1* genotype was a risk factor for ESCD compared with *ALDH2*2/2*2* genotype. Although individuals with *ALDH2*1/2*1* have a strong alcohol metabolism ability, if the alcohol consumption is beyond the metabolism ability the alcohol becomes a dangerous factor for ESCD.

Most reports have indicated that alcohol increases the risk of ESSC in drinkers with *ALDH2*2/2*2* genotype. This leads us to speculate that alcohol will also increase the risk of ESCD in drinkers with *ALDH2*2/2*2* genotype. However, our result did not confirm this association. The reason may be related to the very low frequency of residents who drink alcohol, in particular who indulge in heavy drinking, in Feicheng County, where the people's living standard is relatively low and the majority of farmers cannot afford to drink wine[21,22].

The main finding in the study was an interaction between the *ALDH2* genotype and smoking/family history of esophageal cancer in cases of ESCD, indicating that a polymorphism of *ALDH2* is involved in the process of some carcinogen metabolism, and that it is helpful to control alcohol and tobacco consumption in high incidence areas of esophageal cancer to reduce ESCD and other esophageal diseases.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignant tumors, and alcohol consumption is a major risk factor for esophageal cancer. Acetaldehyde dehydrogenase (ALDH) is related to the risk of ESCC, and esophageal squamous cell dysplasia(ESCD) is one of precancerous lesions, so it is necessary to study the relationship of ALDH and ESCD.

Research frontiers

Most previous reports have indicated that alcohol increases the risk of ESSC in drinkers with *ALDH2*2/2*2* genotype. This leads to speculation that alcohol will also increase the risk of ESCD in drinkers with *ALDH2*2/2*2* genotype. However, this association was not found in this study, but interactions between the *ALDH2* genotype and smoking/family history of esophageal cancer were found in cases of ESCD.

Innovations and breakthroughs

Most reports of case-control studies have only consisted of one case-group of esophageal cancer and one normal control group, and few reports based on population screening data found an association of *ALDH2* with ESCD. In this study, the data of a screening survey for esophageal lesions in a high incidence area of esophageal cancer were used and the association of genetic polymorphisms of *ALDH2* with ESCD in this area was determined.

Applications

It was found that *ALDH2* genotype has interactions with smoking/family history of esophageal cancer for ESCD cases, indicating that a polymorphism of *ALDH2* is involved in the process of some carcinogen metabolism; so it is necessary to control alcohol and tobacco consumption in high incidence areas of esophageal cancer to reduce ESCD and other esophageal diseases.

Terminology

Alcohol is first oxidized by alcohol dehydrogenase to acetaldehyde which is then oxidized to ALDH. The *ALDH2* gene is composed of 13 exons residing on chromosome 12. Deficiency at ALDH2 is a dominant trait that is caused by a single Glu to Lys amino acid substitution at residue 487, a change that can be attributed to a G-to-A transition (dbSNP: rs671) in exon 12 of the gene. The *ALDH2* alleles encoding the active and inactive subunits are termed "*ALDH2*1*" and "*ALDH2*2*" respectively, and *ALDH2*2* exhibits functional polymorphism that is associated with a lower rate of alcohol dependence.

Peer review

This is a well-designed and well-organized study of acetaldehyde dehydrogenase 2 polymorphisms and the risk of esophageal squamous cell dysplasia.

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BRIEF ARTICLE

Radiofrequency ablation in the treatment of small hepatocellular carcinoma: A meta analysis

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Abstract

AIM: To evaluate survival and recurrence after radiofrequency ablation (RFA) for the treatment of small hepatocellular carcinoma (HCC) using a meta-analysis.

METHODS: Literature on RFA *vs* surgical resection for the treatment of small HCC published between January 1990 and December 2008 was retrieved. A meta-analysis was conducted to estimate pooled survival and recurrence ratios. A fixed or random effect model was established to collect the data.

RESULTS: The differences in overall survival at 1-year, 3-years and at end of follow-up were not statistically significant between the RFA and surgery groups ($P > 0.05$). There were no differences in 1-year and 3-year recurrences between the RFA and surgery groups ($P > 0.05$). However, recurrence in the RFA group was lower than that in the surgery group up to the end of follow-up ($P = 0.03$). Survival was not significantly different. There was a significant difference in recurrences at the end of follow-up after RFA compared with surgical resection.

CONCLUSION: RFA did not decrease the number of overall recurrences, and had no effect on survival when compared with surgical resection in a selected group of patients.

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Key words: Hepatectomy; Hepatocellular carcinoma; Meta-analysis; Radiofrequency ablation; Recurrence; Survival

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INTRODUCTION

The preferred treatment for hepatocellular carcinoma (HCC) is surgical resection which has a good long-term effect. In recent years, radiofrequency ablation (RFA) has emerged as the latest oriented treatment, especially for HCC, and has become an important treatment following surgical resection and has established its place in the treatment algorithm of liver tumors. The treatment of HCC in patients with chronic liver disease is a major challenge. With the intention of avoiding hepatic failure which can appear after hepatic resection, percutaneous ablative treatments have been proposed. RFA ablation has progressively reached consensus due to its efficacy, tolerability and low-risk[1]. RFA is much less invasive, involves a short hospital stay and has an extremely low associated mortality; however, long-term results are difficult to ascertain, because the majority of reports concern evaluation of the percentage of success in terms of tumor necrosis and few data are available on the overall

and disease-free survival of patients[2-6]. Clear evidence is still needed for RFA to be accepted as an alternative to surgery for resectable HCC on cirrhosis. Few studies have focused on a comparison between the results of surgery and RFA. In order to reduce research bias and differences, we used a meta-analysis to compare survival and recurrences following RFA compared with surgical resection for the treatment of small HCC. This article may provide a reference for clinical practice.

MATERIALS AND METHODS

Data accrual

We carried out an exhaustive Medline, PubMed, CBM and CNKI search of the world literature comparing survival and recurrences following RFA compared with surgical resection for the treatment of small HCC, between the period January 1990 to December 2008 using the key words (radiofrequency, radio-frequency or radio frequency), (surgical resection or hepatectomy) and (liver or hepatic or hepatocellular) in English, French, German, Italian, Spanish, Danish, Dutch, Korean and Chinese. All abstract supplements from published literature were searched manually. Relevant papers were also identified from the reference lists of previous papers which were obtained through the search, and from abstracts from recent international meetings.

In the case of overlap between 2 reports, only the most detailed report was included. Only series with a minimum follow-up of 12 mo were included. Reports about treatments obtained with noncommercial electrodes and treatments with palliative intent (intentional partial debulking) were excluded. When appropriate, authors were contacted to obtain more details about the cases they reported.

In addition, we chose some Chinese articles, as there are many patients with small HCC in China. A good meta-analysis requires these data.

Data extraction and quality assessment

Data were extracted by two or three independent observers using standardized forms. The recorded data included the number of patients, overall survival and recurrence. The quality of all selected articles was ranked in accordance with the score of the non-randomized controlled clinical trial quality evaluation standard (Table 1).

Study selection criteria

Inclusion criteria for this study were as follows: (1) A solitary HCC smaller than 5 cm in diameter or multiple (no more than three) HCC smaller than 5 cm in total diameter; (2) No extrahepatic metastasis; (3) No radiologic evidence of invasion into the major portal/hepatic vein branches; (4) Good liver function with Child-Pugh Class A or B, with no history of encephalopathy, ascites refractory to diuretics or variceal bleeding; (5) No previous treatment of HCC; (6) Patient should be suitable for treatment with either surgical resection or RFA; and (7) No recurrences where no tumor was found by spiral computed tomography and serum α-fetoprotein level when assessed every 3 mo after treatment during the follow-up period.

Statistical analysis

Meta-analysis was performed using fixed-effect or random-effect methods, depending on the absence or presence of significant heterogeneity. Statistical heterogeneity between trials was evaluated by the Cochran χ^2 test and was considered significant when $P < 0.10$. In the absence of statistically significant heterogeneity, the Mantel-Haenszel method in the fixed-effect model was used for the meta analysis. Otherwise, the DerSimonian and Laird method in the random-effect model was selected.

The odds ratio (OR) with 95% confidence interval (CI) was used to assess treatment efficacy. The combined result was an average OR and 95% CI weighted according to the standard error of the OR of the trial. $P < 0.05$ was considered statistically significant. We used funnel plots to assess the publication bias, and tested for funnel plot asymmetry using Egger's test and Begg's test. All analyses were performed with STATA version 9.0 (Stata Co., College Station, TX, USA) and Review Manager version 4.2.2 (RevMan, Cochrane Collaboration, Oxford, England).

RESULTS

Description of included trials in the meta-analysis

According to exclusion and selected criteria of historical data, 10 studies were selected for the meta analysis, including 787 cases of RFA and 735 cases of surgical resection. However, one publication[7] was removed, because the number of cases continued to expand in another publication[8]. Among the 10 articles selected, 4 (40%) were from China, and corresponded to the high incidence of Hepatitis B virus-associated HCC in China. The characteristics of the 10 clinical trials included are shown in Table 1.

Meta-analysis

The comparison of survival and recurrence following RFA *vs* surgical resection for the treatment of small HCC using the meta-analysis is shown in Figures 1-3 [8-17].

Survival during follow-up 1 year after treatment: The χ^2 test of heterogeneity was highly significant ($P = 0.95$). Accordingly, a fixed-effect model was used. There was no difference in the 1-year overall survival rate between the RFA group (87.9%) and the surgical resection group (88.6%) with a combined OR of 0.94 (95% CI: 0.65 to 1.36, $P = 0.75$, Figure 1).

Survival during follow-up 3-year after treatment: The χ^2 test of heterogeneity was highly significant ($P = 0.0002$). Accordingly, a random-effect model was used. There was no difference in the 3-year overall survival rate between the RFA group (62.5%) and the surgical resection group (63.6%) with a combined OR of 0.92 (95% CI: 0.56 to 1.51, $P = 0.73$, Figure 2A).

Table 1 Outcome data and methodological quality of studies included in the meta-analysis

Author	Yr	Study design	RFA (cases)	Hepatectomy (cases)	Journal	Quality evaluation score[1]
Peng *et al*[8]	2008	Retrospective study	251	183	*Zhongguo Shiyong Waike Zazhi*	7
Vivarelli *et al*[9]	2004	Cohort study	58	40	*Ann Surg*	7
Zhang *et al*[10]	2007	Retrospective study	15	29	*Disan Junyi Daxue Xuebao*	7
Zhou *et al*[11]	2007	Retrospective study	47	40	*Gandan Waike Zazhi*	7
Guglielmi *et al*[12]	2008	Retrospective study	109	91	*J Gastrointest Surg*	7
Montorsi *et al*[13]	2005	Cohort study	79	79	*J Gastrointest Surg*	7
Hong *et al*[14]	2005	Cohort study	55	93	*J Clin Gastroenterol*	9
Wakai *et al*[15]	2006	Retrospective study	21	85	*World J Gastroenterol*	7
Cho *et al*[16]	2005	Retrospective study	99	61	*Korean J Hepatol*	9
Gao *et al*[17]	2007	Retrospective study	53	34	*Zhongguo Yixue Yingxiang Jishu Zazhi*	9

[1]The score from the non-randomized controlled clinical trial quality evaluation standard. RFA: Radiofrequency ablation.

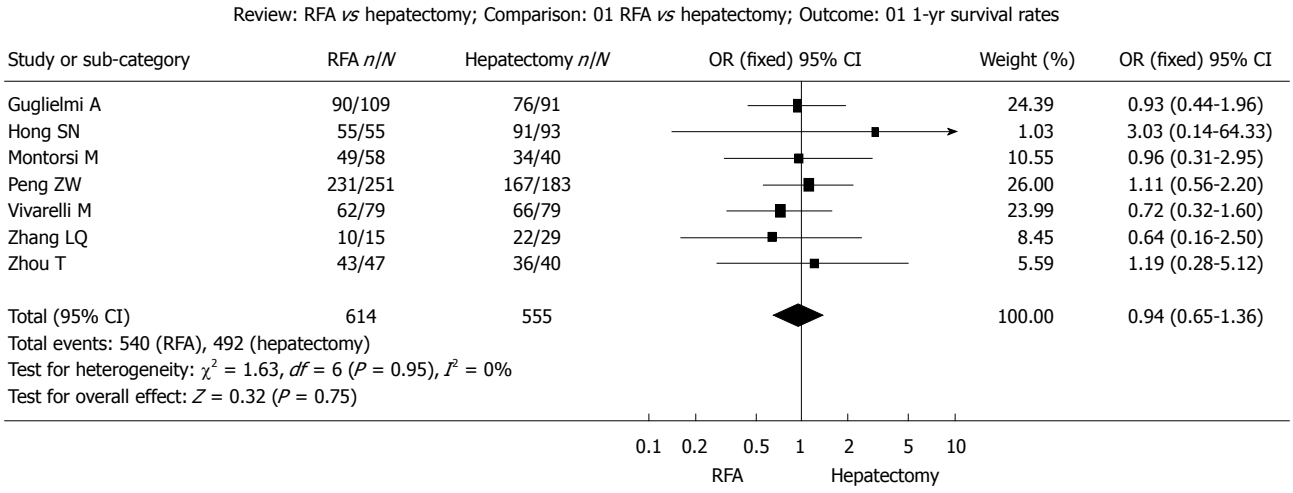

Figure 1 Fixed effect model of odds ratio for survival during follow-up 1-year after treatment: Radiofrequency ablation *vs* hepatectomy. RFA: Radiofrequency ablation.

Survival up to the end of the follow-up period: The χ^2 test of heterogeneity was highly significant ($P < 0.0001$). Accordingly, a random-effect model was used. There was no difference in overall survival rate at the end of follow-up after treatment with RFA (57.4%) compared with surgical resection (60.9%) with a combined OR of 0.82 (95% CI: 0.48 to 1.39, $P = 0.46$, Figure 2B).

Recurrence during follow-up 1-year after treatment: The χ^2 test of heterogeneity was highly significant ($P = 0.07$). Accordingly, a fixed-effect model was used. There was no difference in recurrence rate during follow-up 1-year after treatment between the RFA group (20.6%) and the surgical resection group (20.9%) with a combined OR of 0.96 (95% CI: 0.69 to 1.33, $P = 0.80$, Figure 3A).

Recurrence during follow-up 3-year after treatment: The χ^2 test of heterogeneity was highly significant ($P < 0.0001$). Accordingly, a random-effect model was used. There was no difference in recurrence rate during follow-up 3-years after treatment between the RFA group (59.4%) and the surgical resection group (60.4%) with a combined OR of 1.19 (95% CI: 0.63 to 2.27, $P = 0.59$, Figure 3B).

Recurrence up to the end of the follow-up period: The χ^2 test of heterogeneity was highly significant ($P = 0.0005$). Accordingly, a random-effect model was used. The recurrence rate up to the end of the follow-up period was significantly higher in the RFA group (66.7%) than in the surgical resection group (52.9%) with a combined OR of 1.73 (95% CI: 1.04 to 2.87, $P = 0.03$, Figure 3C).

Sensitivity analysis and publication bias

Publication bias may exist when no significant findings remain unpublished, thus artificially inflating the apparent magnitude of an effect.

Survival and recurrences following RFA or surgical resection for the treatment of small HCC were calculated by the fixed-effect model and random-effect model, respectively. The results were similar and the combined results were highly reliable.

Funnel plots of the study results are shown in Figure 4A-F. The funnel plots on survival and recurrence following RFA or surgical resection for the treatment of small HCC showed basic symmetry, which suggested no publication bias.

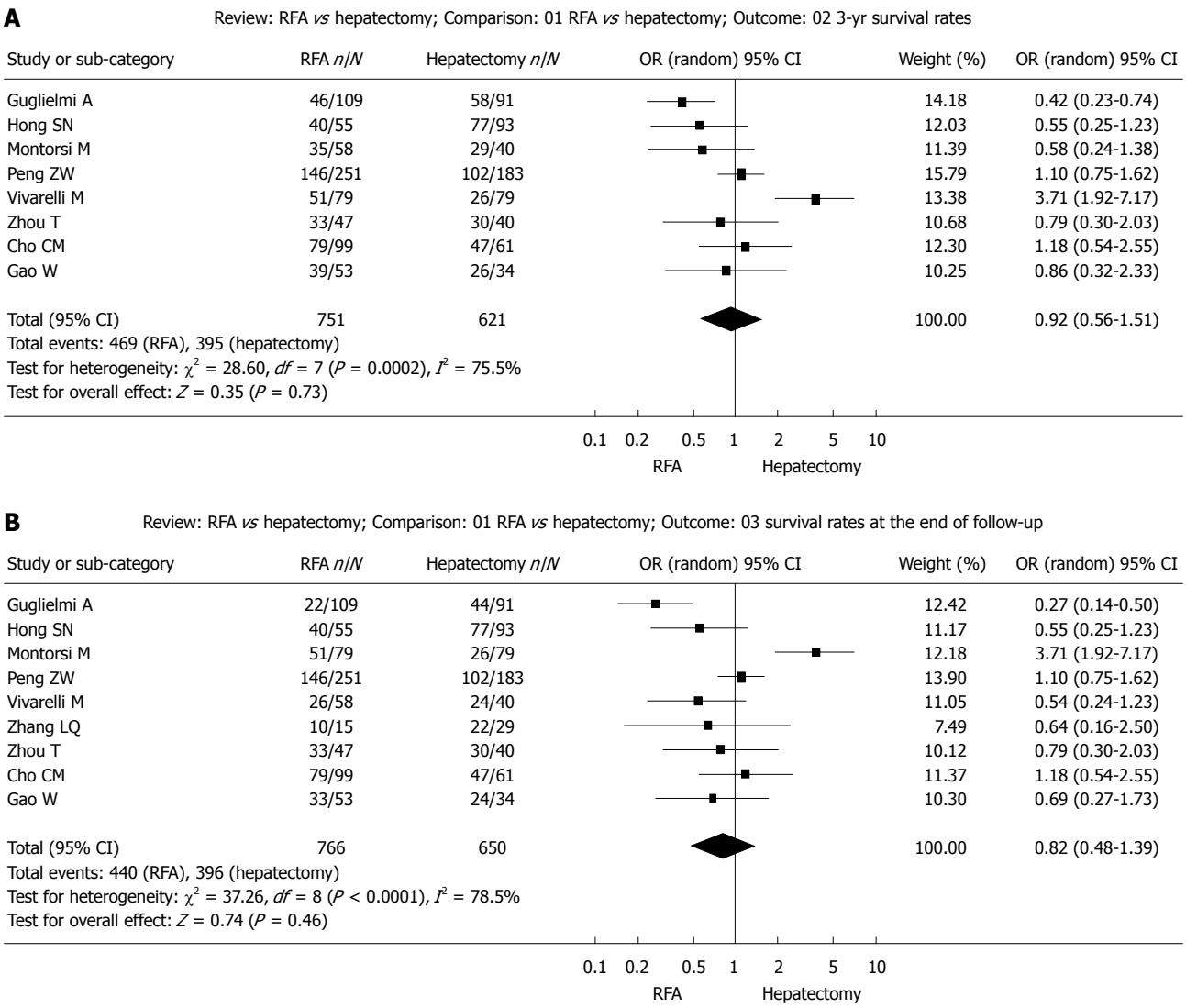

Figure 2 Random effect model of odds ratio for survival of follow-up 3-year (A) and at the end of follow-up (B) after treatment: Radiofrequency ablation *vs* hepatectomy. RFA: Radiofrequency ablation.

DISCUSSION

Hepatocellular carcinoma (HCC) is one of most common malignant tumors of the liver. According to the general condition of patients, tumor location and size and liver function status, surgery can include radical tumor resection, or liver surgery such as local excision. However, there are factors that limit the use of surgical resection. RFA is a relatively new treatment and is now performed more widely, because it results in large coagulated necrosis of the tumor, requires fewer treatment sessions, and achieves higher survival rates[18,19].

RFA has the potential to enhance the long-term survival rate of liver cancer patients worldwide and is of significant importance[20]. Research has indicated that more than 90% of the tumor can be completely destroyed and tumor recurrence *in situ* is effectively inhibited following RFA, which also achieved satisfactory short-term efficacy[21]. Long-term survival following RFA treatment was satisfactory in liver cancer patients as was liver function in those with A-class[22]. The efficacy of RFA was also shown to be related to Child-Pugh grading[23]. Compared with surgery, RFA did not cause significant liver function damage, had a lower rate of complications and was more affordable in terms of treatment costs. The results of this study showed that RFA did not decrease overall recurrences, but had no effect on survival in comparison with surgical resection (i.e. compared with surgical resection, RFA showed no significant difference in the short-term survival rate).

This review has some limitations. Funnel plots can be suggestive of publication bias with lack of negative small RCTs. However, a firm conclusion about bias is difficult to reach as the asymmetry of the funnel plot is minimal. In addition, funnel plots can show asymmetry for reasons other than publication bias. Therefore, our pooled OR might be an overestimate of the true effect. Due to data constraints, this meta-analysis could not analyze the quality of life score and was unable to carry out stratified analyses of other possible confounding factors. If the method is to be more effective, then larger samples and randomized controlled studies with longer

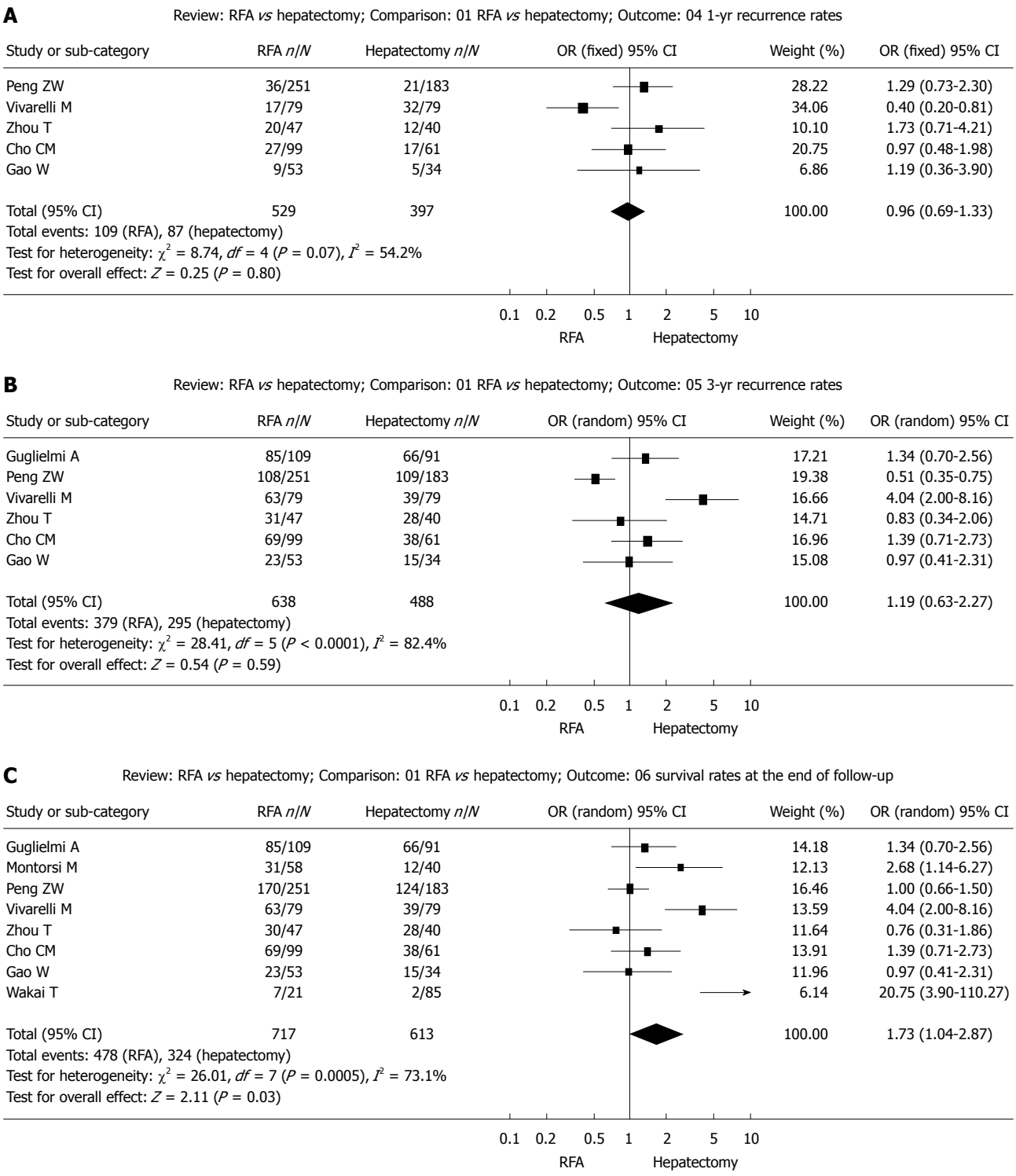

Figure 3 Random effect model of odds ratio for Recurrence of follow-up 1-year (A) and 3-year (B) and the end of follow-up (C) after treatment: Radiofrequency ablation *vs* hepatectomy. RFA: Radiofrequency ablation.

follow-up are required[24]. Chinese article should also be chosen, because there are many patients with small HCC in China. A good meta-analysis requires these data. However, the conclusions of this study also need more detailed data to confirm the results. The search language was limited. The integrity of the data was affected to a certain extent.

In conclusion, with the development of RFA, when conditions permit and under technically assured circumstances, RFA can be performed percutaneously, laparoscopically or during laparotomy, and can partially replace surgical resection. For patients who do not have the opportunity or are unwilling to accept surgical treatment, RFA is an acceptable means of palliative care.

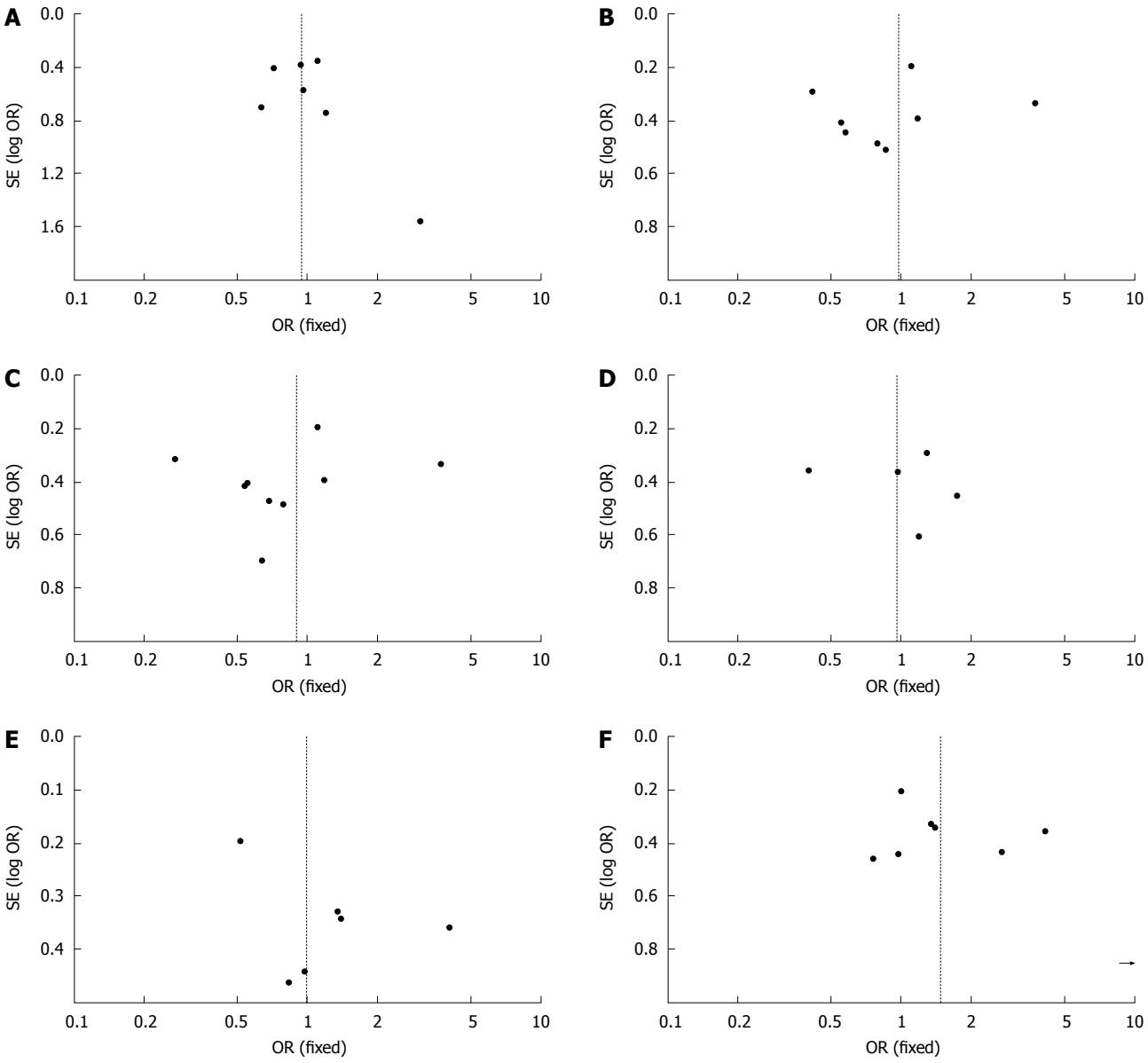

Figure 4 Funnel plots. A: 7 articles in the meta-analysis of survival during follow-up 1-year after treatment; B: 8 articles in the meta-analysis of survival during follow-up 3-years after treatment; C: 9 articles in the meta-analysis of survival up to the end of follow-up; D: 5 articles in the meta-analysis of recurrence during follow-up 1-year after treatment; E: 6 articles in the meta-analysis of recurrence during follow-up 3-years after treatment; F: 8 articles in the meta-analysis of recurrence up to the end of follow-up.

COMMENTS

Background

Over the last decade, radiofrequency thermal ablation (RFA) has established its place in the treatment algorithm of liver tumors. This meta-analysis was designed to evaluate survival and recurrence following RFA for the treatment of small hepatocellular carcinoma (HCC).

Research frontiers

The study evaluated survival and recurrence following RFA for the treatment of HCC using a meta analysis of all relevant controlled studies.

Innovations and breakthroughs

The authors made a comprehensive search of studies dealing with small HCC treated with RFA. The studies were analyzed to determine survival and recurrence after RFA in these patients.

Applications

RFA is an effective technique for the treatment of small HCC and offers an alternative treatment method. This meta-analysis shows that RFA did not decrease overall recurrences, but had no effect on survival in comparison with surgical resection in a selected group of patients. Larger samples and randomized controlled studies with longer follow-up are required.

Peer review

This is an interesting report of RFA *vs* surgical resection for HCC.

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BRIEF ARTICLE

Impact of human leukocyte antigen mismatching on outcomes of liver transplantation: A meta-analysis

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Author contributions: Lan X designed the study, wrote the paper and reviewed all articles for inclusion with Pu CL and Guo CB; Zhang MM performed the final consensus review and the final data analysis; all authors contributed to this paper.

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Abstract

AIM: To assess the effect of human leukocyte antigen (HLA) mismatching on liver graft outcome and acute rejection from a meta-analysis of available cohort studies.

METHODS: Articles in PubMed/MEDLINE, EMBASE and the Cochrane database from January 1970 to June 2009, including non-English literature identified in these databases, were searched. Only studies comparing HLA or sub-phenotype matching with mismatching were extracted. The percentage of graft survival was extracted by "Engauge Digitizer" from survival curves if the raw data were not displayed. A meta-analysis was performed when at least 3 studies provided data.

RESULTS: Sixteen studies met the inclusion criteria. A lower number of HLA mismatches (0-2 *vs* 3-6) did reduce the incidence of acute rejection (relative risk: 0.77, $P = 0.03$). The degree of HLA mismatching (0-2 *vs* 3-6) had no significant effect on 1-year [hazard ratio (HR): 1.04, $P = 0.68$] and 5-year (HR: 1.09, $P = 0.38$) graft survival. In sub-phenotype analysis, the degree of HLA-A, B and DR mismatching (0 *vs* 1-2) had no significant effect on 1-year and 5-year graft survival, either. The HRs and *P*-values were 0.95, 0.71 (HLA-A, 1-year); 1.06, 0.60 (HLA-A, 5-year); 0.77, 0.16 (HLA-B, 1-year); 1.07, 0.56 (HLA-DR, 1-year); 1.18, 0.23 (HLA-DR, 5-year), respectively.

CONCLUSION: The results of this systematic review imply that good HLA compatibility can reduce the incidence of acute rejection in spite of having no influence on graft outcomes. To obtain a short recovery time and minimize rejection post transplantation, HLA matching studies should be considered before the operation.

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Key words: Human leukocyte antigen; Mismatching; Liver transplantation; Meta-analysis; Graft rejection

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Lan X, Zhang MM, Pu CL, Guo CB, Kang Q, Li YC, Dai XK, Deng YH, Xiong Q, Ren ZM. Impact of human leukocyte antigen mismatching on outcomes of liver transplantation: A meta-analysis. *World J Gastroenterol* 2010; 16(27): 3457-3464 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i27/3457.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i27.3457

INTRODUCTION

In the past 2 decades, deaths and other complications of organ transplantation have decreased significantly as a re-

sult of improvements in anesthesiology and surgical techniques. In addition, development of immunosuppressive agents and new organ preservation solutions have been shown to play a role in the improved survival rate. However, acute or chronic rejection remains the most important reason of graft failure, especially for patients who suffer from mismatching of human leukocyte antigen (HLA).

The role of HLA matching between donor and recipient in organ transplant rejection and survival has been widely studied and proven to increase graft survival after kidney, heart, and other organ transplantation and to reduce the incidence of acute or chronic rejection[1-6]. In contrast, major histocompatibility complex analysis is not routinely performed in liver transplantation because its importance remains controversial, with different groups reporting disparate results. It was reported that some populations of patients gained benefit from high degrees of HLA matching[7-14]. Concern has been voiced about possible increased likelihood of recurrence of primary disease with good HLA compatibility[15-25].

We therefore performed a systematic review and meta-analysis on the efficacy of HLA mismatching in all published controlled clinical trials on the outcomes of liver transplantation.

MATERIALS AND METHODS

Search strategy

Relevant articles that were published between January 1970 and June 2009 in PubMed/MEDLINE, EMBASE and the Cochrane database, including the non-English literature were identified. The search strategy used the following single text words and combinations: living donor liver transplantation (LDLT), liver transplantation (LT), orthotopic liver transplantation (OLT), human leukocyte antigen (HLA), major histocompatibility complex (MHC), histocompatibility, matching and mismatching. Reference lists of relevant articles were cross checked for other potentially relevant articles.

Selection of trials and quality of the studies

Three separate authors (Lan X, Pu CL and Guo CB) independently reviewed and evaluated all articles for inclusion, which were classified as randomized control trial (RCT), controlled trial (CT), or descriptive study. After the initial article selection, the article dataset was reviewed and updated to capture any articles published between the final consensus review and the final data analysis (Zhang MM). Only cohort studies were indentified because of a lack of RCT.

The scoring system was adapted from Stahl, the Cochrane Collaboration and others[26-29]. This system suits not only RCT but CT or other studies well: (1) Was the trial design clearly stated? (2) Selection bias questions: Was the Patient selection process clearly stated? If the trial was an RCT, were patients randomly allocated to the therapeutic intervention? Were patients and clinicians blinded to the intervention? If the trial was not an RCT, were confounders controlled for? If the trial design was case control were matching procedures clearly described and implemented? Were patient recruitment procedures clearly described? Were the intervention and control groups selected similarly? (3) Performance bias questions: Was the intervention clearly described? Was intervention clearly measured? (4) Attrition bias questions: Were patients followed up? Were they followed up for 2 or more explicitly defined intervals? If patients were lost/dropped out other than because of death, were they accounted for? Were all outcome measures captured at the declared follow-up intervals? (5) Detection bias questions: Were the outcome measures clearly described? Was measurement of the outcome measures blinded? (6) Were appropriate statistical methods used? Were *P*-values clearly stated? Was life table analysis provided, *etc*.; and (7) Was the presentation of data adequate, for example, in the article were endpoints clearly defined i.e. graft survival, patient survival, duration of follow-up, re-transplantation rate, *etc*.? Were survival curves provided or were sufficient data to construct survival curves provided, were donor and recipient variables clearly defined and presented?

These questions were placed on a 3 point scale: unclear/inadequate (0), adequate (1), good (2). Articles were considered for inclusion if their summary score exceeded 30.

Data extraction

Graft loss was measured by hazard ratio (HR) and rejection was measured by relative risk (RR) at 1-year and 5-year in every study by 2 independent reviewers, reconciling any differences by consensus or when in doubt referring it to a third reviewer (Zhang MM) for arbitration. Graft survival rate was extracted for calculating corresponding HR using the formula recommended by Parmar *et al*[30]. Data was extracted by the software "Engauge 4.0" from survival curves if it was not shown in articles directly. Donor/recipient HLA compatibility for HLA class Ⅰ (A and B), and HLA class Ⅱ (DR) was measured as the number of mismatches, locus-specific (0 to 2 mismatches) and overall for the A, B, and DR loci (0 to 6 mismatches).

Meta-analysis

Both HR and RR were compared between 0 with 1-2 mismatches for each locus (mismatches of the HLA-A, B and C loci respectively) and 0-2 with 3-6 mismatches for overall HLA-A, B and DR loci. Comparability of the studies included in each pooled analysis was confirmed by examination of the $\chi^2 Q$ (expressed as a *P*-value) and I^2 statistics of heterogeneity. Statistical heterogeneity was defined as $P < 0.10$ or $I^2 > 50\%$. Lack of over-influence of one individual study to pooled estimates was confirmed by serial omission of each study and examination of the resulting estimate. To account for potential differences that were evident clinically but not identified by statistical tests, random effects models were used for each outcome measure. All statistical analyses were performed using Review Manager 5.0 which was a new program for determining HR.

Table 1 Contents of included studies

Author	Location	Immunosuppression	Number of patients	Contents
Meyer *et al*[9]	France	Cyclosporine, methylprednisolone and azathioprine	162	HLA-A, B and DR (5-yr graft survival); HLA-DR (1- and 5-yr graft survival)
Jakab *et al*[10]	American	NS	631	HLA-A, B and DR (1- and 5-yr graft survival); HLA-A and HLA-B (5-yr graft survival)
Neumanna *et al*[8]	Germany	Cyclosporine, azathioprine and prednisolone	836	HLA-A, B and DR (1- and 5-yr graft survival and rejection); HLA-A and HLA-DR (1- and 5-yr graft survival); HLA-B (1-yr graft survival)
Hashimoto *et al*[11]	Japan	Cyclosporine, methylprednisolone and azathioprine	50	HLA-A, B and DR (1- and 5-yr graft survival)
Langrehr *et al*[12]	Germany	Cyclosporine, azathioprine and prednisolone	165	HLA-A, B and DR (1- and 5-yr graft survival and rejection)
Suehiro *et al*[13]	Japan	Tacrolimus and Steroids	104	HLA-A, B and DR (1- and 5-yr graft survival and rejection)
Harihara *et al*[14]	Japan	Tacrolimus and Steroids	85	HLA-A, B and DR (rejection)
Balan *et al*[7]	American	Cyclosporine, prednisone, and azathioprine or tacrolimus	799	HLA-A, B and DR (1- and 5-yr graft survival); HLA-A (5-yr graft survival)
Sugawara *et al*[16]	Japan	Tacrolimus and methylprednisolone	113	HLA-DR (1-yr graft survival)
Doran *et al*[22]	Germany	NS	446	HLA-A, B and DR (1- yr graft survival); HLA-A and HLA-B (1-yr graft survival)
Poli *et al*[15]	Italy	Cyclosporine, azathioprine and tacrolimus	814	HLA-DR (5-yr graft survival)
Yagihashi *et al*[18]	American	Cyclosporine, azathioprine and tacrolimus	347	HLA-A, HLA-B and HLA-DR (1-yr graft survival)
Nikaein *et al*[21]	American	Cyclosporine and prednisone	701	HLA-A, B and DR (1-yr graft survival); HLA-A (1- and 5-yr graft survival); HLA-B and HLA-DR (1-yr graft survival)
Markus *et al*[20]	American	NS	527	HLA-A (5-yr graft survival); HLA-DR (1-yr graft survival)
Donaldson *et al*[19]	Britain	Cyclosporine, azathioprine	466	HLA-A, B and DR (1-yr graft survival and rejection); HLA-A and HLA-B (1-yr graft survival)
Knechtle *et al*[17]	American	NS	324	HLA-A, B and DR (1-yr graft survival); HLA-A, HLA-B and HLA-DR (1-yr graft survival)

NS: Not specified; HLA: Human leukocyte antigen.

RESULTS

Results of the article selection are described in Figure 1. 1568 potentially relevant articles were identified in the search. The abstracts of these studies were reviewed by 2 independent investigators. One thousand four hundred and forty-two did not meet inclusion criteria as their summary score was less than 30. Publications eligible for analysis included 16 articles: 2 prospective studies[7,8] and 14 retrospective cohort studies[9-22]. Non RCTs were included in our studies. In 4 studies acute rejection rates were compared clearly between 0-2 mismatches and 3-6 mismatches of HLA[8,12-14]. That is too say, specific data could only be extracted in these 4 articles. In 10 and 8 studies 1-year and 5-year survival rates, respectively, were compared between 0-2 mismatches and 3-6 mismatches of HLA[7-10,12,13,15,17,19,21,22]. In 6 and 5 studies 1-year and 5-year survival rates, respectively, were compared or could be extracted from survival curves between 0 mismatches and 1-2 mismatches of the HLA-A epitope[7,8,10,17-22]. In 9 and 5 studies 1-year and 5-year survival rates, respectively, were compared or could be extracted from survival curves between 0 mismatches and 1-2 mismatches of the HLA-DR epitope[8,9,16-22]. In 6 studies 1-year survival rates were compared between 0 mismatches and 1-2 mismatches of the HLA-B epitope[8,17-19,21,22]. Although 0 mismatches of the HLA-B epitope were compared with 1-2 mismatches in 5-year survival rates in 3 articles, the statistical heterogeneity was $P = 0.004$ and $I^2 = 82\%$ in the meta-analysis. Hence, the HR of the HLA-B epitope in 5-year survival rates was not included in our discussion. Details of these studies are described in Table 1.

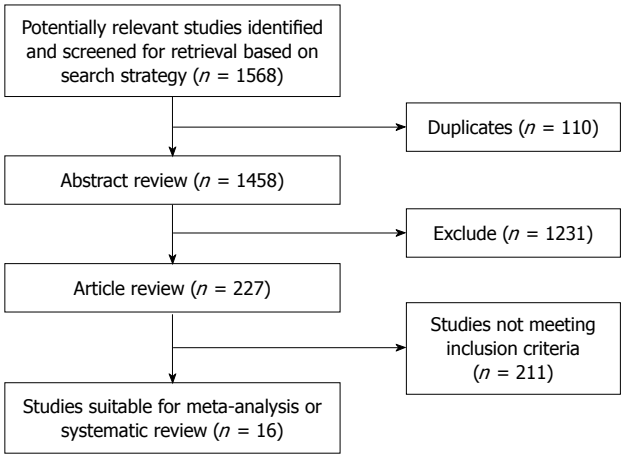


Figure 1 Selection of articles.

Table 2 Methodological quality of the controlled trials

Study	Selection criteria specified	Study design	Score	Other causing of death report	Dropouts explained	Funding
Meyer C	Yes	RCS	30	No	No	NS
Jakab SS	Yes	RCS	32	Yes	Yes	NS
Neumanna UP	Yes	PCS	31	No	No	NS
Morioka D	Yes	RCS	30	No	No	NS
Langrehr JM	Yes	RCS	33	Yes	Yes	NS
Suehiro T	Yes	RCS	30	Yes	No	NS
Harihara Y	Yes	RCS	30	No	No	NS
Vijayan B	Yes	PCS	35	Yes	Yes	NS
Sugawara Y	Yes	RCS	30	Yes	No	NS
Doran	Yes	RCS	30	No	No	NS
Poli F	Yes	RCS	31	No	No	NS
Yagihashi A	Yes	RCS	30	No	No	NS
Afzal N	Yes	RCS	33	No	No	NS
Markus BH	Yes	RCS	32	No	Yes	NS
Donaldson P	Yes	RCS	32	No	Yes	NS
Knechtle SJ	Yes	RCS	31	No	Yes	NS

RCS: Retrospective cohort studies; NS: Not specified.

The methodological quality of the studies was assessed using a validated tool as described above (Table 2).

Meta-analysis of HLA epitope

HLA-A, B and DR (0-2 mismatches *vs* 3-6 mismatches): In the studies included in the meta-analysis, a total of 4260 patients were included in 10 articles (1-year graft survival) and 3180 patients were included in 8 articles (5-year graft survival). No differences between 0-2 mismatches and 3-6 mismatches of HLA-A, B, and DR epitopes were seen in terms of 1-year graft survival [HR: 1.04, 95% confidence interval (CI): 0.86-1.25, $P = 0.68$] and 5-year graft survival (HR: 1.09, 95% CI: 0.90-1.32, $P = 0.38$, Figure 2).

HLA-A epitopes (0 mismatch *vs* 1-2 mismatches): Of the studies included in the meta-analysis, there were a total of 2049 patients in 6 articles (1-year graft survival) and 2138 patients in 5 articles (5-year graft survival). No differences between 0 mismatch and 1-2 mismatches of the HLA-A epitopes were seen in terms of 1-year graft survival (HR: 0.95, 95% CI: 0.72-1.25, $P = 0.71$) and 5-year graft survival (HR: 1.06, 95% CI: 0.85-1.34, $P = 0.60$, Figure 2).

HLA-B epitopes (0 mismatch *vs* 1-2 mismatches): A total of 1969 patients were included in 6 articles (1-year graft survival). No differences between 0 mismatch and 1-2 mismatches of the HLA-B epitopes were seen in terms of 1-year graft survival (HR: 0.77, 95% CI: 0.53-1.11, $P = 0.16$, Figure 2).

HLA-DR epitopes (0 mismatch *vs* 1-2 mismatches): A total of 2688 patients were included in 9 articles (1-year graft survival) and 2175 patients were included in 5 articles (5-year graft survival). No differences between 0 mismatch and 1-2 mismatches of the HLA-DR epitopes were seen in terms of 1-year graft survival (HR: 1.07, 95% CI: 0.84-1.37, $P = 0.56$) and 5-year graft survival (HR: 1.18, 95% CI: 0.90-1.54, $P = 0.23$, Figure 2).

HLA epitopes and acute rejection

A total of 1268 patients were included in 4 articles (acute rejection within 3 mo after transplantation). Significant differences between 0-2 mismatches and 3-6 mismatches of HLA-A, B and DR epitopes were seen in terms of acute rejection (RR: 0.77, 95% CI: 0.61-0.97, $P = 0.03$, Figure 2).

DISCUSSION

This is the first systematic review and meta-analysis on the effect of HLA mismatching in short and long term liver graft outcome and acute rejection. We identified and analyzed 16 unique cohort studies and all HLA locus-specific analyses were performed by standard lymphocytotoxicity tests with confirmation by polymerase chain reaction, with HLA-A, B and DR locus mismatches being compared. The results clearly showed that a lower number of HLA mismatches (0-2 *vs* 3-6) did reduce the incidence of acute rejection. The degree of HLA mismatching (0-2 *vs* 3-6) had no significant effect on 1-year and 5-year graft survival. Furthermore, we found no difference between 0 mismatches and 1-2 mismatches in 1-year and 5-year graft survival of HLA-A, HLA-B and HLA-DR on subgroup analysis.

The role of HLA matching between donor and recipient in liver transplant rejection and graft survival has been determined in some cohort studies and there still is no consensus view[7-15,31]. This systematic review analyzed the different data of various studies and has given our own results. However, the main objective in performing this analysis was to assess the necessity of donor-recipient HLA matching before liver transplantation.

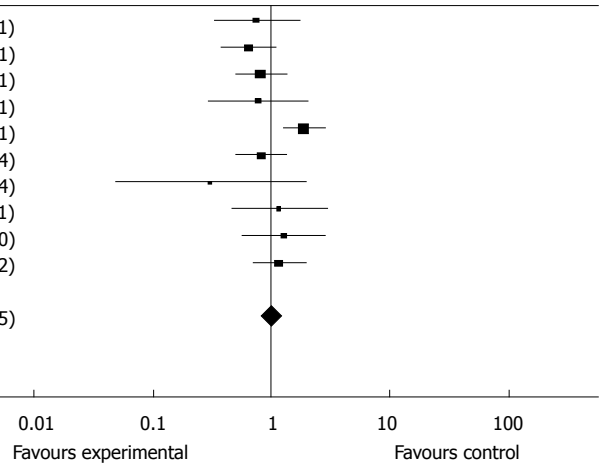
As the role of HLA matching between donor and recipient in organ transplant rejection and survival had been proven to increase graft survival after kidney and heart transplantation, it has been debated whether these matches affected the outcomes of the liver graft similarly. In liver transplantation, organ allocation relies mostly on ABO blood group, recipients' body weight, and clinical urgency, and the outcome of liver grafts relies mostly on complications after transplantation; HLA matching is usually not taken into account and the literature is inconsistent on the role of this parameter. In fact, any complications after transplantation are associated with graft outcome and rejection. Liver artery thrombosis, venous thromboembolic complications, seventh-day syndrome, primary graft nonfunction, and serious infection can decrease survival[32-36]. Compared to HLA mismatching, these complication are more important for long term graft survival.

Although the liver graft was considered to be a kind of immune-free organ, in our meta-analysis a lower number of HLA mismatches (0-2 *vs* 3-6) did reduce the incidence of acute rejection. It has become clear in recent years that mismatching of HLA in liver grafts led to endothelialitis induced by the recipient's natural killer cells and so rejection was instigated. The association of acute rejection with

A

Study or subgroup	Weight (%)	Hazard ratio Exp [(O-E)/V], fixed, 95% CI
Nikaein A 1994	5.3	0.76 (0.34, 1.71)
Morioka D 2007	12.0	0.65 (0.38, 1.11)
Doran TJ 2000	16.2	0.83 (0.52, 1.31)
Langrehr JM 2006	3.9	0.78 (0.30, 2.01)
Donaldson P 1993	22.5	1.90 (1.28, 2.81)
Jakab SS 2007	15.3	0.83 (0.52, 1.34)
Knechtle SJ 1993	1.0	0.31 (0.05, 1.94)
Suehiro T 2005	4.1	1.17 (0.47, 2.91)
Neumann UP 2002	5.6	1.27 (0.58, 2.80)
Balan V 2008	14.1	1.17 (0.72, 1.92)
Total (95% CI)	100.0	1.04 (0.86, 1.25)

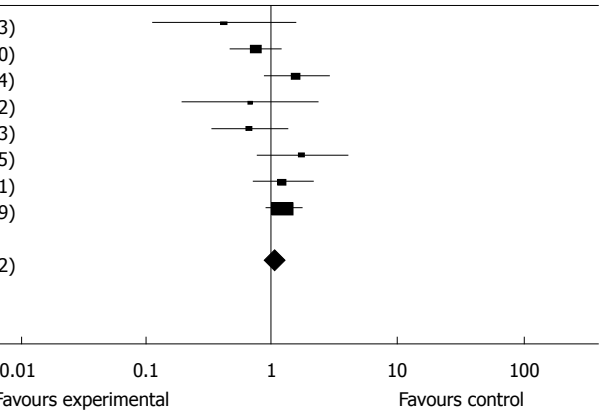
Heterogeneity: χ^2 = 16.95, *df* = 9 (*P* = 0.11); I^2= 47%
Test for overall effect *Z* = 0.42 (*P* = 0.68)



B

Study or subgroup	Weight (%)	Hazard ratio Exp [(O-E)/V], fixed, 95% CI
Meyer C 1997	2.2	0.42 (0.12, 1.53)
Morioka D 2007	17.6	0.76 (0.48, 1.20)
Poli F 2001	10.7	1.57 (0.87, 2.84)
Langrehr JM 2006	2.4	0.67 (0.19, 2.32)
Jakab SS 2007	8.0	0.67 (0.34, 1.33)
Suehiro T 2005	5.7	1.76 (0.78, 3.95)
Neumann UP 2002	13.2	1.24 (0.73, 2.11)
Balan V 2008	40.1	1.25 (0.92, 1.69)
Total (95% CI)	100.0	1.09 (0.09, 1.32)

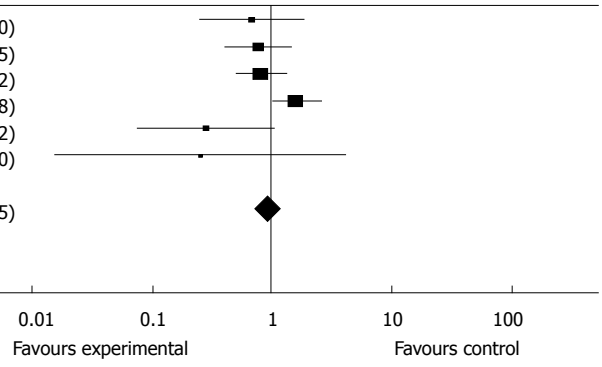
Heterogeneity: χ^2 = 10.77, *df* = 7 (*P* = 0.15); I^2= 35%
Test for overall effect *Z* = 0.88 (*P* = 0.38)



C

Study or subgroup	Weight (%)	Hazard ratio Exp [(O-E)/V], fixed, 95% CI
Yagihashi A 1992	7.5	0.70 (0.26, 1.90)
Nlkaeln A 1994	19.6	0.78 (0.42, 1.45)
Jakab SS2007	33.3	0.82 (0.51, 1.32)
Donaldson P 1993	33.9	1.61 (1.00, 2.58)
Knechtle SJ 1993	4.6	0.29 (0.08, 1.02)
Neumann UP 2002	1.0	0.26 (0.02, 4.10)
Total (95% CI)	100.0	0.95 (0.72, 1.25)

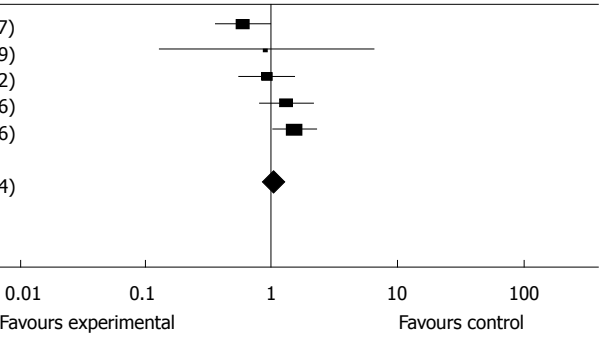
Heterogeneity: χ^2 = 10.18, *df* = 5 (*P* = 0.10); I^2= 49%
Test for overall effect *Z* = 0.37 (*P* = 0.71)



D

Study or subgroup	Weight (%)	Hazard ratio Exp [(O-E)/V], fixed, 95% CI
Balan V 2008	22.9	0.60 (0.37, 0.97)
Neumann UP 2002	1.4	0.91 (0.13, 6.49)
Jakab SS 2007	21.1	0.93 (0.56, 1.52)
Markus BH 1988	21.8	1.32 (0.81, 2.16)
Nikaein A 1994	32.9	1.52 (1.02, 2.26)
Total (95% CI)	100.0	1.06 (0.85, 1.34)

Heterogeneity: χ^2 = 5.13, *df* = 3 (*P* = 0.16); I^2= 42%
Test for overall effect *Z* = 0.53 (*P* = 0.60)



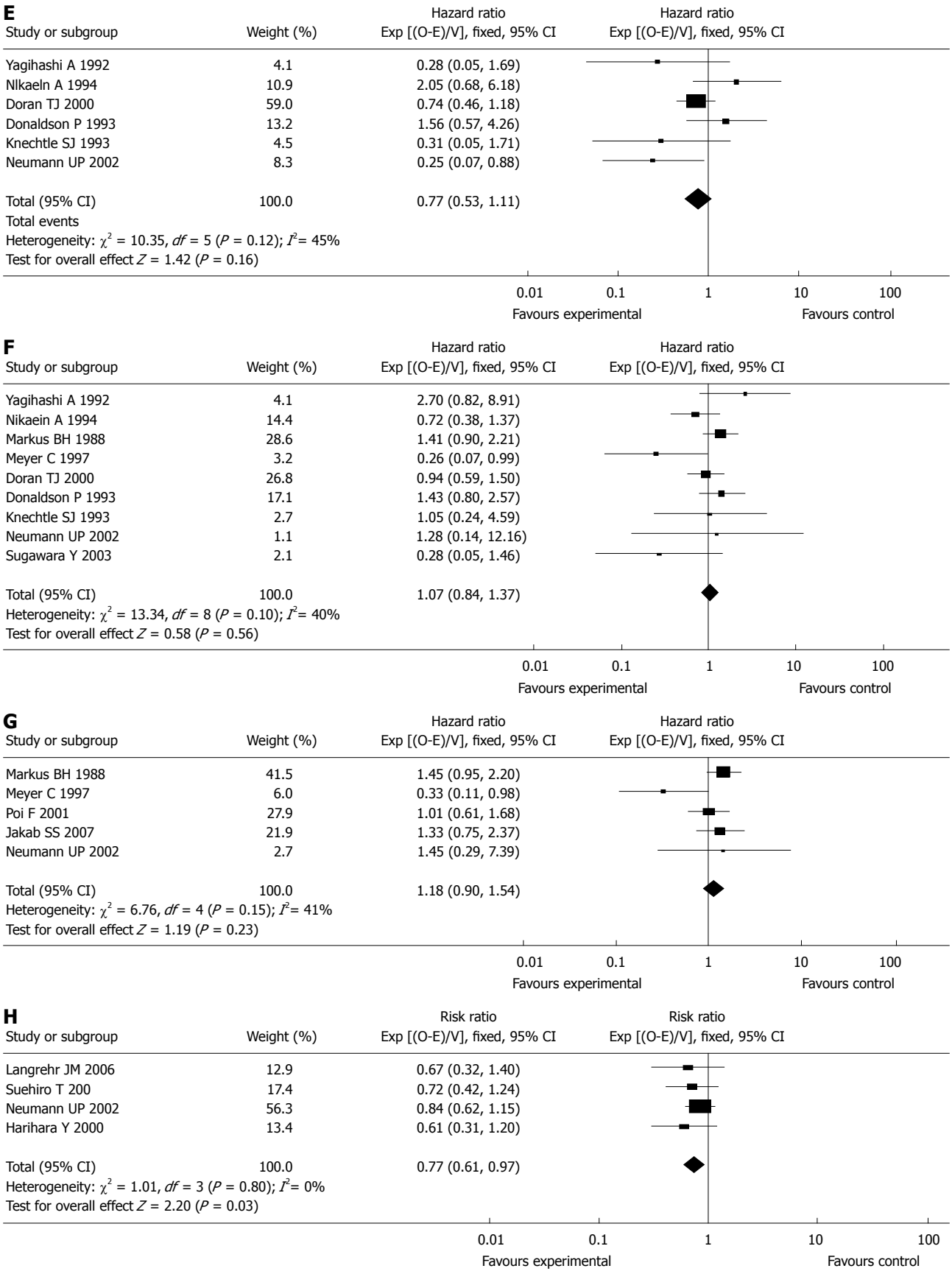


Figure 2 Meta-analysis of cohort trials comparing the effect of different mismatches of human leukocyte antigen epitopes on graft survival and acute rejection. A: 0-2 *vs* 3-6 mismatches of human leukocyte antigen (HLA)-A, B, DR epitopes on 1-year graft survival; B: 0-2 *vs* 3-6 mismatches of HLA-A, B, DR epitopes on 5-year graft survival; C: 0 *vs* 1-2 mismatches of HLA-A epitopes on 1-year graft survival; D: 0 *vs* 1-2 mismatches of HLA-A epitopes on 5-year graft survival; E: 0 *vs* 1-2 mismatches of HLA-B epitopes on 1-year graft survival; F: 0 *vs* 1-2 mismatches of HLA-DR epitopes on 1-year graft survival; G: 0 *vs* 1-2 mismatches of HLA-DR epitopes on 5-year graft survival; H: 0-2 *vs* 3-6 mismatches of HLA-A, B and DR epitopes on acute rejection.

3 other risk factors (cold ischemia time greater than 15 h, pretransplantation elevation of aspartate transaminase, and older donor age) are less readily explained, but suggest that nonallogeneic and allogeneic immunological injury may be related. Both a long cold ischemia time and older donor age predispose an allograft liver to injury shortly after transplantation, which evokes immunological reactions that are not necessarily triggered by allogeneic differences.

Although a meta-analysis may provide a high level of scientific evidence, it is important to realize the limitations of interpreting results of meta-analyses. One major limitation to the meta-analysis is that inferences are based on aggregate analysis of relatively heterogeneous studies. We acknowledge the potential heterogeneity of combining studies from different centers in different geographic locations with different treatment protocols. In our systematic review, results obtained from each study were considered to be homogeneous (heterogeneity test was $P > 0.10$ and $I^2 < 50\%$ in all available studies) in spite of there being no RCTs in this meta-analysis. Although we did not investigate through meta-regression any differences in the use of immunosuppressants or differences in study centers, the treatment protocols were nearly the same: cyclosporine or tacrolimus, azathioprine and prednisolone and no mycophenolate mofetil were used (Table 1).

Additionally, some studies did not report results with the measures that we chose for data extraction. It is the second limitation we must deal with. Survival rates under 1-year or 5-year were extracted by special software from survival curves if they was not shown in articles directly. We did not even obtain any data from some cohort studies, but including or excluding these articles also did not affect our conclusions.

The length of post transplantation follow-up was another limitation of many of the trials that we analyzed. Although most trials reported follow-up of some patients up to 5 years or even longer, some reported follow-up only to 1 year or 6 mo. Long-term graft survival, including HBV, HCV and hepatocellular carcinoma recurrence, may only become apparent or more pronounced after many years of post liver transplantation follow-up, and hence we may have underestimated the mortality in our study. In other words, we may have overestimated the role of HLA mismatching in liver graft loss.

Despite these limitations, our meta-analysis suggests that a lower number of HLA mismatching did reduce the incidence of acute rejection. The degree of HLA mismatching had no significant effect on 1-year and 5-year graft survival. Performing good donor-recipient HLA matching appears to be associated with a reduction in the incidence of acute rejection. Thus to obtain a shorter recovery time and avoid more rejection post transplantation, HLA matching examinations should be considered before surgery.

COMMENTS

Background

The role of human leukocyte antigen (HLA) matching between donor and recipient in organ transplant rejection and survival has been widely studied and proven to increase graft survival and to reduce the incidence of acute or chronic rejection. In contrast, major histocompatibility complex analysis is not routinely performed in liver transplantation because its importance remains controversial.

Research frontiers

Different groups have reported disparate results on the effect of HLA matching: some patients acquired benefit from high degrees of HLA matching but concern has been voiced about a greater likelihood of recurrence of primary disease with good HLA compatibility.

Innovations and breakthroughs

This is the first systematic review and meta-analysis on the effect of HLA mismatching in short and long term liver graft outcome and acute rejection. Importantly, the authors have some different conclusions compared to traditional views. Good donor-recipient HLA matching appears to be associated with a reduction in the incidence of acute rejection although there is no effect on 1-year and 5-year survival rates.

Applications

The percentage of graft survival was extracted by "Engauge Digitizer" from survival curves if the raw data was not presented. All statistical analyses were performed using Review Manager 5.0 which was a new program for determining HR.

Peer review

The authors aimed to assess the effect of HLA mismatching in liver graft outcome and acute rejection from available cohort studies by a systematic review and meta-analysis. The design of the study is rational and reliable, and the statistical methods used are appropriate. The article is also well organized. The conclusion may provide reliable and valuable information for clinical practice.

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BRIEF ARTICLE

Precise prediction model and simplified scoring system for sustained combined response to interferon-α

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Abstract

AIM: To establish a predictive algorithm which may serve for selecting optimal candidates for interferon-α (IFN-α) treatment.

METHODS: A total of 474 IFN-α treated hepatitis B virus e antigen (HBeAg)-positive patients were enrolled in the present study. The patients' baseline characteristics, such as age, gender, blood tests, activity grading (G) of intrahepatic inflammation, score (S) of liver fibrosis, hepatitis B virus (HBV) DNA and genotype were evaluated; therapy duration and response of each patient at the 24th wk after cessation of IFN-α treatment were also recorded. A predictive algorithm and scoring system for a sustained combined response (CR) to IFN-α therapy were established. About 10% of the patients were randomly drawn as the test set. Responses to IFN-α therapy were divided into CR, partial response (PR) and non-response (NR). The mixed set of PR and NR was recorded as PR+NR.

RESULTS: Stratified by therapy duration, the most significant baseline predictive factors were alanine aminotransferase (ALT), HBV DNA level, aspartate aminotransferase (AST), HBV genotype, S, G, age and gender. According to the established model, the accuracies for sustained CR and PR+NR, respectively, were 86.4% and 93.0% for the training set, 81.5% and 91.0% for the test set. For the scoring system, the sensitivity and specificity were 78.8% and 80.6%, respectively. There were positive correlations between ALT and AST, and G and S, respectively.

CONCLUSION: With these models, practitioners may be able to propose individualized decisions that have an integrated foundation on both evidence-based medicine and personal characteristics.

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Key words: Chronic hepatitis B; Interferon-α; Patient selection; Predictive model; Scoring system; Treatment outcome

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INTRODUCTION

Chronic hepatitis B (CHB) is one of the most refractory diseases that mankind faces and is a serious global public health problem. Hepatitis B virus (HBV) infection often leads to acute or chronic hepatitis B, hepatocellular carcinoma (HCC) and other complications. Of approximately 350 million carriers of HBV worldwide, about 1 million die from chronic complications, such as cirrhosis, HCC or both every year[1]. According to Liu *et al*[2], there is a 9% rate of HBV surface antigen (HBsAg) in the general population in China. In the United States, there are an estimated 1.25 million HBV carriers[3]. Currently, available antiviral options can be divided into 2 types, interferon-α (IFN-α) and nucleoside/nucleotide analogues, including conventional IFN-α, pegylated IFN-α (PEG-IFN), lamivudine, adefovir dipivoxil, entecavir, telbivudine, *etc*. IFN-α is one of the major choices; however, some factors greatly hinder its wide range of applications. First of all, the expense of antiviral therapy is considerable in either underdeveloped or developing countries; in addition, there are several side effects, such as fatigue, flu-like syndrome and others; ultimately, the most important aspect is that only a proportion of patients may achieve a response after therapy[4,5]. Thus, antiviral therapy is not generally accepted by patients.

Predicting the efficacy of IFN-α is crucial before attempting treatment for CHB patients. Some factors, such as HBV genotype A or B, lower viral load, higher serum alanine aminotransferase (ALT) levels, higher grading (G) of intrahepatic inflammation, and lower staging (S) of liver fibrosis, have been identified to be the predictors of the outcome of IFN-α therapy in HBV e antigen (HBeAg)-positive patients[3,6,7]. Female gender, short course of disease, having mild liver fibrosis, having good compliance with therapy, absence of co-infection and an early virological response at the 12th wk also indicate a good therapeutic outcome[8]. Sometimes, however, patients do not have all the "positive" predictors. They may also have one or more "negative" predictors. For example, a patient infected by HBV of genotype C, has a high viral load, accompanied by high ALT and high G. Is he suitable for IFN-α treatment? As pointed out by the European Association for the Study of the Liver, the HBV genotype has a poor individual predictive value, and currently, genotype alone should not define the choice of treatment[7]. These types of issues may be challenging for both practitioners and patients. Owing to the trials that had rigorous designs, many patients have benefited from evidence-based medicine developed in the last several decades. However, evidence-based medicine aims at the resolution of issues which came from individuals with a common background whereas individual information is not always taken into account. The current research aims at making a sensible decision that has an integrated foundation in both evidence-based medicine and personal characteristics.

We therefore conducted the present study to determine (1) baseline predictive factors for the response to IFN-α; (2) what was the relationship between these predictive factors; (3) whether a predictive algorithm for IFN-α treatment of CHB can be derived from these factors; and (4) what was the efficacy of the model.

MATERIALS AND METHODS

Patients

During the period between July 2005 and November 2008, all HBeAg-positive CHB patients were followed up for their response to IFN-α if they initially started IFN-α treatment in our Liver Division, the 174th Hospital of the PLA, the Traditional Chinese Medicine Hospital of Xiamen, Zhongshan Hospital Xiamen University, Xiamen, or Macheng Hospital, Hubei. Patients were recruited according to the guidelines in China[8], and were administrated with 5 MU of conventional IFN-α every other day for 24 wk or longer. The patients' baseline information was collected, including age, gender, blood tests, G, S, HBV DNA, genotype, *etc*. Several studies have indicated the potential benefits of extended duration of IFN-α or PEG-IFN therapy regarding a sustained response[9,10] or suppression of chronic complications[11]. Thus, duration was also recorded for balancing the effect of therapy span. Patients were excluded if they had HCC on presentation or other concomitant diseases including hepatitis A, C or D virus infection, autoimmune hepatitis, Wilson's disease, primary biliary cirrhosis and alcoholic liver disease. Patients with the following conditions were also excluded: pregnancy, mental disorders (such as severe depression), uncontrolled epilepsy, alcohol abuse, narcotic abuse, uncontrolled autoimmune disorders, decompensated liver cirrhosis, symptomatic heart disease, neutrophil count below 1.0×10^9/L and/or platelet count below 50×10^9/L before treatment, had received or were receiving any other form of established treatment for CHB. Finally, 474 patients were included in the current study. For the treatment of HBeAg-positive CHB, a combined response (CR) was defined as ALT levels returning to normal, undetectable HBV DNA, and HBeAg seroconversion; partial response (PR) was defined as ALT levels returning to normal, HBV DNA $< 10^5$ copies/mL, but no seroconversion; whereas non-response (NR) refers to no CR or PR observations[8]. The mixed set of PR and NR was recorded as PR+NR. A sustained response was defined as the response at the 24th wk after cessation of IFN-α treatment.

Monitoring of patients

Patients were followed up every 1-2 mo by monitoring HBsAg status, HBeAg/anti-HBe status, HBV DNA level,

ALT, aspartate aminotransferase (AST), α-fetoprotein (AFP), complete blood count and mental status. Complete blood counts were taken once every 1-2 wk for the first month, then once per month until cessation of treatment. Other tests, such as thyroid function, blood glucose, routine urinalysis, were taken once every 3 mo. For patients who had abnormal thyroid function at baseline, appropriate therapy was initiated, and thyroid function was closely monitored during antiviral therapy. If there was evidence of a depressive disorder or suicidal tendency, treatment was stopped and patients were closely monitored. Ultrasound of the liver was scheduled for patients with AFP levels greater than 20 ng/mL. Patients were suggested to stop IFN-α administration if CR or NR occurred after therapy for 24 wk, or if severe side effects developed during the course of treatment. Patients' choices were also taken into account.

Determination of HBV genotypes and HBV DNA levels

Sera from patients on presentation were taken for the following tests: (1) HBV genotyping performed by the polymerase chain reaction (PCR)-fluorescence detection kit for HBV genotype B, C according to the manufacturer's instructions (Bioselex, Hangzhou, China); and (2) HBV DNA levels were determined by quantitative fluorescence PCR on the ABI 7000 (Applied Biosystems), with a lower limit of detection of 1000 copies/mL. HBV DNA levels below the lower detection limit were regarded as negative for statistical calculations.

Statistical analysis

Statistical analyses were performed using version R 2.8.1 (a language and environment for statistical computing, Vienna, Austria, ISBN 3-900051-07-0, http://www.R-project.org). The inter-variable correlation was determined by the Spearman rank correlation coefficient. The Gini index based on random forest methodology was used to determine whether the identified variables were associated with therapy outcomes. In the present study, the response to IFN-α treatment (dependent variable) was ordinal data. If the independent variable was ordinal data (such as ALT, AST, G, S, *etc.*), Kendall's *tau-b* test was adopted to test the statistical significance between independent variable and dependent variable. For the nominal independent variable (gender, genotype, *etc.*), the Pearson χ^2 test was used.

About 10% of patients were randomly selected as the test set, and the remaining patients were employed as the training set. The predictive model was constructed with a support vector machine (SVM) package for the R platform. Accuracies for CR and PR+NR in the training set and test set were calculated. The above process was repeated 300 times and mean accuracy was calculated. Performance of the constructed predictive algorithm was evaluated by the mean accuracies for CR and PR+NR for the training set and test set. The scoring system for sustained CR (SCR) was derived from our observations (Table 1) with computer-aided minor adjustment according to other data[3,7,12,13]. The weight for every level in each factor was rounded to the nearest integer.

Table 1 Baseline demographic and virological data of the study population

Factor	*n* (range)
Sex (M:F)	345:129
Age (yr)	29.8 (10-58)
ALT (U/L)	250 (16-1908)
AST (U/L)	146 (24-1304)
Genotype (A:B:C)[1]	51:212:211
HBV DNA (log copies/mL)	7.35 (5.00-9.83)
Fibrosis staging, S (0:1:2:3:4)	10:154:157:114:39
Histology activity index, G (1:2: 3:4)	39:215:169:51

Continuous variables are expressed as median (range). [1]B, C refers to genotype B, C, respectively; genotype B and C co-infection and other genotypes were named as A. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBV: Hepatitis B virus.

The area under the curve (AUC) was then calculated for measuring the overall prediction accuracy. A 95% confidence interval (CI) for an AUC was obtained by sampling the 474 patients for 1000 bootstrap samples with the confidence limits calculated as the 2.5th and 97.5th percentiles. The scoring system was assessed by the leave-one-out cross-validation in order to assess the performance of new data[14]. To ease clinical employment of the SCR score, cut-off values were determined by maximizing the Youden index, i.e. sensitivity + specificity - 1, calculated from the receiver operating characteristic curves analysis. Accuracy of using the optimal cut-off values was assessed by the sensitivity, specificity, predictive values and likelihood ratios. Their 95% CIs were obtained from 1000 bootstrap samples. The cut-off values were also cross-validated by the leave-one-out method.

RESULTS

Demographics

A total of 474 CHB patients were enrolled. The baseline demographics, liver function tests, liver biochemistry, histology data and virological data are listed in Table 1. As shown in Table 2, the ratios of CR, PR and NR at the 24th wk after cessation of IFN-α therapy were 34.4%, 45.1% and 20.5%, respectively. It should be pointed out that genotype A in the current research refers to co-infection of genotype B and C, and other genotypes beside B and C.

Patients' factors and treatment factor for the response to IFN-α therapy

As shown in Table 2, female patients had a higher chance of a CR compared to male patients (41.1% *vs* 31.9%, $P < 0.001$; Kendall's *tau-b* test). Genotype B had a preferential effect on CR (45.3% and 25.1% for genotype B and genotype C, respectively, $P < 0.001$, Pearson χ^2 test). ALT and AST had a positive reciprocal relationship with treatment response ($P < 0.001$; Kendall's *tau-b* test) (Table 2).

Table 2 Individual factors of patients with diverse responses at the 24th week after cease of interferon-α therapy

	CR	PR	NR
Sex (M:F)	110:53	163:51	72:25
Age [0-14):(15-24):(25-44):(≥ 45), yr]	3:64:123:5	5:47:148:14	2:21:71:3
ALT [(1-2):(2-3):(3-5):(5-10):(≥ 10), ULN]	7:5:27:75:49	27:41:71:44:31	19:31:31:13:3
AST [(0-1):(1-2):(2-3):(3-5):(5-10):(≥ 10), ULN]	1:22:30:49:43:18	12:76:54:38:24:10	9:50:18:14:6:0
Genotype (A:B:C)	14:96:53	30:101:83	7:15:75
HBV DNA [(5-5.99):(6-6.99):(7-7.99):(8-8.99):(≥ 9), log copies/mL]	21:55:56:26:5	19:65:79:49:2	5:31:37:21:3
Fibrosis staging, S (0:1:2:3:4)	2:54:51:48:8	7:68:71:50:18	1:32:35:16:13
Histology activity index, G (1:2:3:4)	11:71:60:21	22:95:77:20	6:49:32:10
Responses (CR:PR:NR)	163	214	97

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ULN: Upper limit of normal; HBV: Hepatitis B virus; CR: Combined response; PR: Partial response; NR: No response.

Table 3 Inter-variable correlations determined by the Spearman rank correlation coefficient

	Gender	Age	Grading	Staging	ALT	AST	DNA[1]	Genotype	Duration	Y F6 m[2]
Gender	1.00									
Age	0.06	1.00								
Grading	-0.11[a]	0.05	1.00							
Staging	-0.06	0.08	0.74[b]	1.00						
ALT	0.05	-0.01	0.13[b]	0.02	1.00					
AST	-0.13[b]	-0.04	0.25[b]	0.17[b]	0.73[b]	1.00				
DNA[1]	-0.09	0.04	-0.04	-0.10[a]	0.07	0.09[a]	1.00			
Genotype	0.09[a]	0.00	-0.01	0.01	0.09	0.03	-0.12[b]	1.00		
Duration	0.05	-0.03	-0.02	0.03	0.00	-0.04	0.03	0.02	1.00	
Y F6 m	0.05	-0.01	-0.03	0.00	-0.39[b]	-0.35[b]	0.06	0.25[b]	-0.07	1.00

[a]$P < 0.05$, [b]$P < 0.01$; [1]log copies/mL; [2]Response after 6 mo of follow-up. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Inter-variable correlations

The correlations between variables were determined by the Spearman rank correlation coefficient. As shown in Table 3, there were positive reciprocal relationships between G and S (0.74, $P < 0.01$), ALT and AST (0.73, $P < 0.01$). Baseline ALT (0.39, $P < 0.01$), AST (0.35, $P < 0.01$) and genotype (0.25, $P < 0.01$) had a substantial predictive effect on the sustained response. The correlations between G and S, ALT and AST are illustrated in Figure 1A and B.

Multivariate analysis for factors associated with the response to IFN-α therapy

Stratified by duration of IFN-α therapy, Gini index analysis showed that baseline predictors, from highly significant to least significant, were ALT, HBV DNA in log copies/mL, AST, genotype, S, G, age and gender (Table 4).

Predictive algorithm for the SCR to IFN-α therapy

Based on SVM, a predictive model was developed for the SCR to IFN-α therapy. According to the established model, the accuracies for SCR and PR+NR respectively were 86.4% and 93.0% for the training set, 81.5% and 91.0% for the test set.

Predictive scoring system for the SCR to IFN-α therapy

Based on our data provided in Table 2 with computer-aided minor adjustment according to other data[3,7,12,13], a predictive scoring system was developed for the SCR to IFN-α therapy. The odds ratio of SCR score was 15.25 (95% CI: 9.65-24.68, $P < 0.001$) indicating that the scoring system had an excellent prediction performance. By optimizing with the Youden's index, the optimal cut-off for the prediction of SCR was 169. This cut-off had good sensitivity and specificity and had been accurately validated by the leave-one-out validation (Table 5). The AUCs were as high as 0.797 (95% CI: 0.773-0.812) for SCR prediction (Figure 2A). The odds ratio of SCR according to the scoring system is depicted in Figure 2B.

Table 4 Significance of baseline factors for sustained combined response to interferon-α therapy

Variable	CR	PR	NR	Mean decrease accuracy	Mean decrease Gini
ALT	2.242	0.860	2.611	1.363	42.806
Duration	0.536	0.599	0.251	0.507	36.340
HBV DNA	0.603	0.742	0.015	0.553	35.713
AST	1.045	-0.243	0.955	0.502	35.488
Genotype	1.101	1.083	3.095	1.269	30.462
Staging	0.737	-0.058	-0.147	0.205	29.400
Grading	-0.033	0.330	-0.035	-0.182	23.283
Age	-0.069	0.094	0.160	0.050	20.944
Gender	-0.125	0.478	-0.084	0.186	14.396

CR: Combined response; PR: Partial response; NR: No response; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBV: Hepatitis B virus.

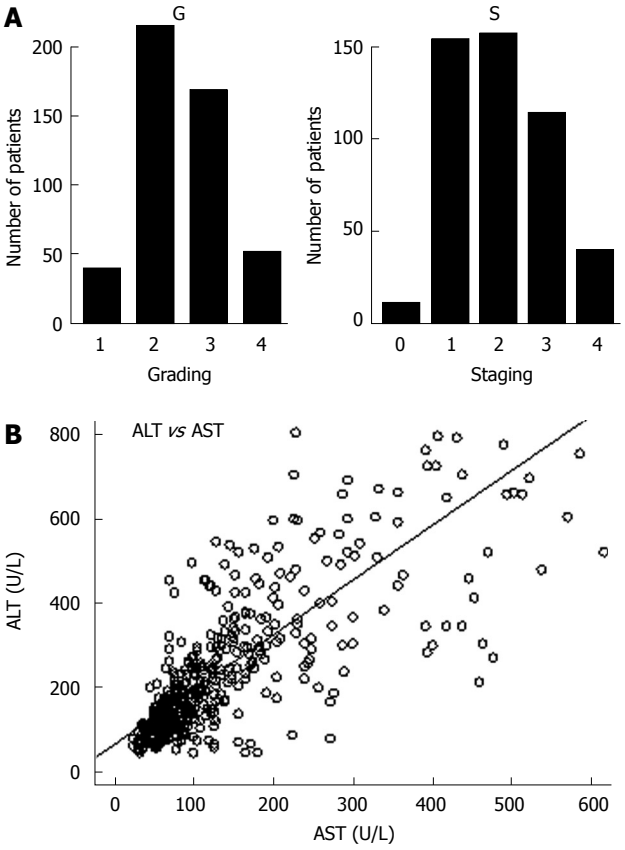

Figure 1 Inter-variable correlations between grading and staging, alanine aminotransferase and aspartate aminotransferase. A: Positive correlation between grading (G) and staging (S); B: Positive correlation between alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Table 5 Optimal cut-off values by maximizing the Youden index and their accuracies for the sustained combined response score derived from whole study population and validated with leave-one-out cross-validation

	Value	95% CI
Total study population		
Optimal cut-off	169	
Sensitivity (%)	78.79	71.93-84.33
Specificity (%)	80.58	75.81-84.61
Positive predictive value (%)	68.42	61.50-74.61
Negative predictive value (%)	87.68	83.34-91.00
Positive likelihood ratio	4.06	3.19-5.16
Negative likelihood ratio	0.26	0.20-0.36
Odds ratio	15.25	9.65-24.68
Accuracy (%)	79.96	76.12-83.31
Youden index	0.594	0.5913-0.5961
AUC	0.797	0.773-0.812
Leave-one-out cross-validation		
Optimal cut-off	169	
Sensitivity (%)	78.18	71.28-83.80
Specificity (%)	79.94	75.11-84.02
Positive predictive value (%)	67.54	60.61-73.78
Negative predictive value (%)	87.28	82.89-90.67
Positive likelihood ratio	3.90	3.08-4.94
Negative likelihood ratio	0.27	0.20-0.37
Odds ratio	14.13	8.98-22.73
Accuracy (%)	79.32	75.45-82.73
Youden index	0.581	0.5787-0.5836
AUC	0.79	0.779-0.807

AUC: Area under the curve.

DISCUSSION

In summary, the main findings and results of the current research include: (1) development of an accurate predictive model for the SCR to IFN-α therapy; (2) deduction of a scoring system for SCR as the data mining method may be unavailable to most the clinicians; (3) identification of positive reciprocal relationships between G and S, ALT and AST; (4) a predictive role of baseline ALT, AST and genotype for SCR; and (5) baseline predictive factors, listed from the greatest to the least significant, are ALT, HBV DNA, AST, genotype, S, G, age and gender.

The predictive factors for the response to IFN-α therapy have been extensively investigated; however, response prediction for individual patients remains uncertain. First, evidence-based medicine aims at a therapeutic strategy for patients with a similar background rather than a given patient. Additionally, a given individual may have "positive" predictive factors and "negative" predictive factors at the same time. Thus, patients or even clinicians may be perplexed by the probability estimation of therapy outcome. This leads to significant profligacy of health resources and a delay in treating patients who need an appropriate antiviral intervention. Fortunately, the present study may facilitate the management of these difficulties. One of the limitations of the current study is that most of the genotypes are B and C, or co-infection of B and C, which is in accordance with the report by Zeng *et al*[15]. There are not enough patients infected by other genotypes of HBV for statistical analysis. Another limitation is that treatment with conventional IFN-α rather than PEG-IFN was evaluated in the present research. Although PEG-IFN was extensively prescribed in developed countries, its application was greatly hindered by its high cost in developing countries. In China, there is great disparity in the prescription costs of IFN-α and PEG-IFN. We cannot recruit enough patients administrated with PEG-IFN for statistical analysis. However, IFN-α and PEG-IFN share the same bioactive molecule *in vivo* and similar baseline predictors[16]. Therefore, using our scoring system, which was easily employed in clinical practice, the response to PEG-IFN therapy may be predicted with reasonable accuracy. If statistical packages were available, higher predictive accuracy could be achieved.

In line with several studies[12,17], genotypes B or C have dramatically different effects on treatment response. Apart from genotypes, the present study also found that increasing HBV DNA levels were associated with a stepwise decrease in the response, which was similar to that of S; in contrast, increasing ALT, AST and G played an opposite role. Patients with higher ALT, AST and G tended to have better outcomes. According to the inter-variable correlation analysis, there were significantly positive reciprocal relationships between ALT and AST, G and S, respectively. The correlation between ALT and AST sounds reasonable, which may be a result of parallel release of intracellular contents after immune injury.

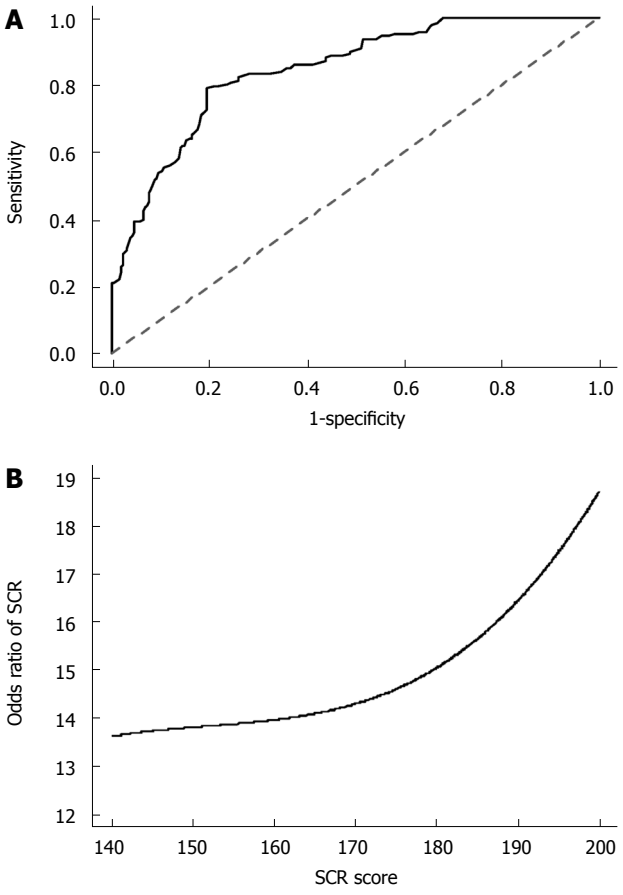

Figure 2 Predictive performance of the scoring system for sustained combined response. A: Receiver-operator characteristics curve of the scoring system for sustained combined response (SCR); B: Odds ratio of the scoring system for SCR.

Repeated intrahepatic immunologic inflammation triggers fibrinogen secretion from hepatic stellate cells and eventually leads to cirrhosis, which may partly explain the correlation between G and S. The performed study indicated the most important predictive factor was baseline ALT, followed by HBV DNA level, AST, genotype, S, G, age and gender. Interestingly, the Gini index of S was slightly higher than that of G, which may be due to the higher variation of G than that of S though they have a high correlation as mentioned before. Baseline AST also had a considerable predictive role for SCR. Although ALT is regarded as a specific index for HBV-induced inflammation, AST rather than ALT manifests a correlation with fibrosis stage[18]. Data by Brook *et al*[19] also indicated AST > 85 U/L is a predictive factor for the response to IFN-α therapy.

We developed a predictive model that was shown to have parallel accuracies for both a training set and test set by adjusting kernel parameters, which ensured that satisfactory sensitivity may be achieved for samples out of the observation pool. In other words, the established model would have a reasonable predictive accuracy for patients who were not enrolled in the current research. In addition, a scoring system for SCR was developed to identify patients who may have SCR if the score was greater or equal to the optimal cut-off value of 169. This score was validated by the stringent leave-one-out statistical analysis with high sensitivity and specificity of 78.2% and 79.9%, respectively, for the prediction of SCR. Using these SCR scores, the practitioner can calculate the prognosis of a patient on presentation, which is important for devising individual management of the patient. The practitioner can also identify very high-risk patients who should be recommended for treatment by nucleoside/nucleotide analogues to obtain good results.

In clinical practice, we are not aware of any predictive score for the SCR to IFN-α therapy in HBeAg-positive CHB patients with the integration of potential predictive factors. Our novel predictive algorithm and SCR score may serve as an excellent reference for clinicians to decide who should undergo IFN-α therapy. With these models, practitioners would be able to propose individualized treatment paradigms that have an integrated foundation in both evidence-based medicine and personal characteristics. It has extensive potential clinical use to identify CHB patients who have a high potential of a SCR to IFN-α therapy. These patients should be suggested to be treated by IFN-α to delay or prevent lethal complications of CHB such as liver cirrhosis and HCC.

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COMMENTS

Background

Predicting the efficacy of interferon-α (IFN-α) is crucial before attempting treatment of chronic hepatitis B (CHB) patients. Though predictors of the responses to IFN-α have been well identified, patients frequently have "positive" and "negative" predictors at the same time. Therefore, it is very difficult to predict the treatment response before IFN-α therapy for a specific patient.

Research frontiers

Evidence-based medicine aims at the resolution of issues which came from individuals with a common background whereas individual information can not always be taken into account. The current research aims at sensible decision-making that has an integrated foundation on both evidence-based medicine and personal characteristics.

Innovations and breakthroughs

The current research successfully integrated the patients' personal characteristics into the foundations of evidence-based medicine. A precise prediction model and a simplified scoring system for a sustained combined response (SCR) to IFN-α were generated.

Applications

It has extensive clinical use to identify CHB patients who have a high potential of a SCR to IFN-α therapy. With these predictive models, practitioners would be able to propose individualized treatments that have an integrated foundation in both evidence-based medicine and personal characteristics.

Peer review

This is an interesting report of a prediction model and simplified scoring system for SCR to IFN.

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CASE REPORT

Anterograde jejunojejunal intussusception resulted in acute efferent loop syndrome after subtotal gastrectomy

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Abstract

Postoperative intussusception is an unusual clinical entity in adults, and is rarely encountered as a complication following gastric surgery. The most common type after gastric surgery is retrograde jejunogastric intussusception, and jejunojejunal intussusception has been rarely reported. We report a case of anterograde jejunojejunal intussusception after radical subtotal gastrectomy with Billroth Ⅱ anastomosis in a 38-year-old Korean woman with early gastric cancer, and include a review of the literature on this unusual complication.

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Key words: Intussusception; Postoperative complications; Gastrectomy

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INTRODUCTION

Postoperative intussusception is an unusual clinical entity in adults, and is rarely encountered as a complication following gastric surgery. The reported incidence is under 0.1% in patients who undergo gastric surgery[1]. The most common type after gastric surgery is jejunogastric intussusception[2]. Jejunojejunal intussusception has been rarely reported, and all previously reported cases have been observed after Roux-en-Y gastro- or esophagojejunostomy. Here, we report a case of anterograde jejunojejunal intussusception that developed at the efferent limb after subtotal gastrectomy with Billroth Ⅱ reconstruction in a woman with early gastric cancer. We also include a review of the literature on this unusual complication. To the best of our knowledge, this is the first case report of jejunojejunal intussusception after partial gastrectomy with Billroth Ⅱ type gastrojejunostomy.

CASE REPORT

A 38-year-old woman was referred to our outpatient clinic for biopsy-proven adenocarcinoma of the stomach. The patient had no significant medical history. On gastroduodenoscopic examination, type Ⅱc early gastric cancer was found at the lesser curvature of the body. Moderately differentiated adenocarcinoma was confirmed by performing a biopsy of the lesion. Staging work-up including abdominal computed tomography (CT) revealed no metastasis.

Radical subtotal gastrectomy with Billroth Ⅱ reconstruction, including complete dissection of the perigastric nodes plus the lymph node along the left gastric and hepatic arteries, was performed. The lesser curvature of the stomach and the duodenal stump were closed with staples. Antecolic, isoperistatic gastrojejunostomy was performed using Albert-Lembert sutures. A nasogastric tube was placed in the remnant stomach.

On the first postoperative day (POD), the patient did not complain of abnormal symptoms. However, on POD 2, she started to complain of recurrent episodes of abdominal colicky pain, which did not subside for over 5 d,

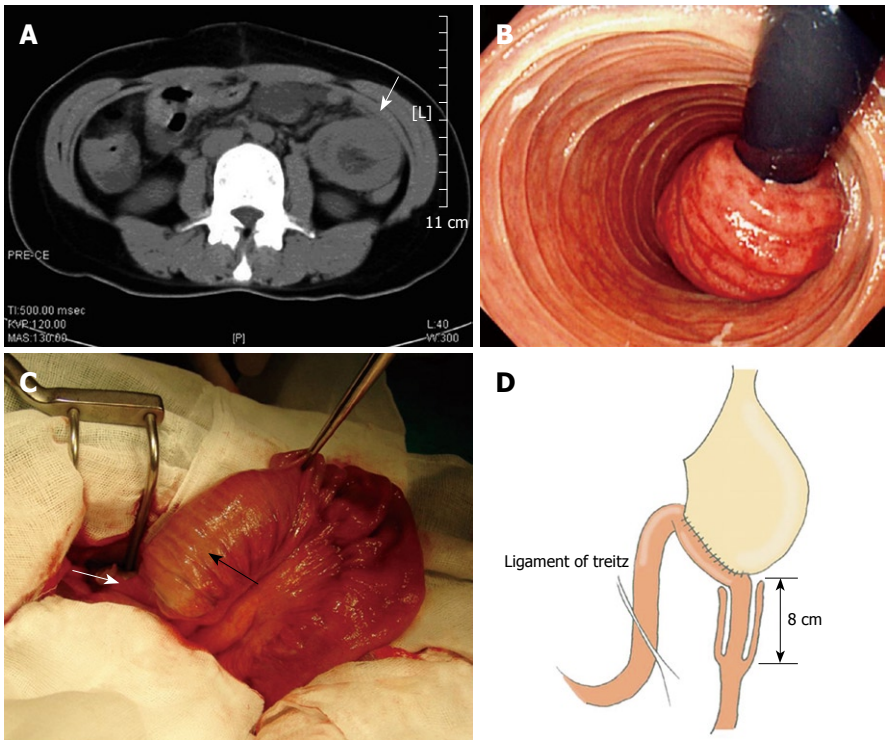

Figure 1 Radiologic, endoscopic and intraoperative findings of anterograde jejunojejunal intussusception. A: Abdominal computed tomography showed a non-homogeneous mass (arrow) in the left upper quadrant of the abdomen; B: A J-turn view of gastrofibroscopic examination demonstrated a congested jejunal mass (intussusceptum); C: Intraoperative findings revealed that the intussusception started just below the gastrojejunostomy anastomosis. The black arrow indicates the intussuscipiens, and the white arrow indicates the intussusceptum; D: Schematic diagram of the intussusception at the efferent-loop.

without a definite cause. During that time, 500-900 mL bile-containing gastric juice was drained *via* the nasogastric tube daily. Serial plain abdominal radiography and laboratory tests showed no remarkable findings. The blood tests were unremarkable except for elevated white blood cell count up to 16000/μL. On POD 6, the patient complained of intermittent abdominal cramping, and abdominal tenderness in the left upper quadrant of the abdomen on physical examination. Non-enhanced CT was carried out and revealed a non-homogeneous jejunal mass below the anastomosis site (Figure 1A). Gastrofibroscopic examination was performed subsequently. The entrance of the efferent loop was narrow and edematous, but the gastrofibroscope was able to pass through the efferent loop. A congested jejunal mass was observed on the J-turn image (Figure 1B). Emergency laparotomy was performed, on suspicion of a jejunojejunal intussusception.

On exploration, an anterograde jejunojejunal intussusception was found at about 5 cm distal to the gastrojejunostomy anastomosis line (Figure 1C and D). Manual reduction was performed carefully and the intussusception came loose subsequently. The proximal jejununal segment (intussusceptum), about 8 cm in length, did not recover from ischemia. The primary gastrojejunostomy anastomosis, which was non-viable, was removed, and a Roux-en-Y gastrojejunostomy was formed. On pathological examination, no identifiable leading point for the intussusception was present. She was discharged without further complications or abnormal symptoms on POD 11 after the second operation.

DISCUSSION

Intussusception is primarily a disease of infancy and childhood, and only about 5% of cases occur in adults[3]. Although childhood intussusception is idiopathic in 90% of cases, adult intussusception has an organic lesion as a leading cause in 70%-90% of cases, and > 50% of the lesions have been reported to be malignant[4,5].

Although postoperative intussusception is a rare clinical entity in both age groups, it is also more common in the pediatric population than in adults. It accounts for 5%-10% of cases of postoperative ileus in infancy and childhood[6], and only 1% of cases in adults[7].

Intussusception is an extremely rare complication after gastric surgery; the incidence is reported to be < 0.1%[1]. Since the first case of jejunogastric intussusception after gastrojejunostomy was reported by Bozzi[8] in 1914, a large number of isolated cases have been reported, and fewer than 200 cases of postoperative intussusception after gastric surgery have been reported in the English-language literature[2]. Retrograde jejunogastric intussusception is the most common type after gastric surgery[9]. Rarer cases of jejunojejunal[10-14], jejunoduodenal[15] or duodenogastric intussusception[16], or intussusception through a Braun anastomosis[17] also have been reported after gastric surgery.

All cases of jejunojejunal intussusception have been observed after Roux-en-Y gastro- or esophagojejunostomy. To the best of our knowledge, this is the first case report of jejunojejunal intussusception after partial gastrectomy with Billroth II type gastrojejunostomy. However, it should be pointed out that jejunojejunal intussusception might not be such a rare problem after Roux-en-Y gastric bypass for morbidly obese patients[18]. Simper *et al*[18] recently have reported 22 cases (0.15%) of postoperative jejunojejunal intussusception after more than 15000 Roux-en-Y gastric bypasses.

In the present case, neither functional nor mechanical causes were identified as leading causes of intussusception. Although most adult intussusceptions are caused

by a definable structural lesion[5], definite anatomical or pathological causes are rarely found in cases following gastric surgery[2]. A variety of postoperative conditions, such as adhesions around the suture lines[3,19], a long intestinal tube[20], increased intra-abdominal pressure[1], shortening of the jejunal mesentery[21], and reverse peristalsis[19,22], have been proposed as possible mechanisms of intussusception after gastric surgery, but none has been confirmed. Functional causes include reverse peristalsis, which is triggered by an anastomosed jejunal loop being irritated by hydrochloric acid, and atonic stomach, which is caused by vagotomy[2].

Diagnosis of postoperative intussusception is difficult in adults. The classic symptom triad of intussusception (pain, palpable mass, and currant-jelly stool) rarely occurs in adults[3]. Furthermore, usual symptoms encountered in acute postoperative intussusception are easily confused with postoperative ileus or adhesion[23,24]. For these reasons, diagnosis was delayed in our case, which rapidly progressed to incarceration and strangulation of the involved efferent limb. Thus, a high index of clinical suspicion is necessary for early diagnosis of this potentially lethal complication. Upper gastrointestinal endoscopy and abdominal CT are highly diagnostic in the setting of urgent, high-level intestinal obstruction[25].

Endoscopic reduction of jejunogastric intussusception has been suggested in a few selected cases[26]; however, this is associated with a significant risk of recurrence[27]. Surgery is the mainstay of treatment in jejunojejunal intussusception, and should be individualized for each patient. Surgical procedures include reduction, resection, and revision of the intussusception and takedown of the previous anastomosis with construction of a new anastomosis, depending on the operative findings. If the affected segment loses viability, resection of the non-viable segment is inevitable. Ozdogan *et al*[13] have suggested that the operation should be conservative, provided that the bowel is viable; manual reduction is the only required treatment, and other preventive measures are not necessary.

We report a case of anterograde jejunojejunal intussusception that caused acute efferent loop syndrome after partial gastrectomy. Acute jejunojejunal intussusception after gastric surgery is an extremely rare clinical entity that requires a high index of clinical suspicion for early diagnosis and prompt surgical management. Thus, intussusception should be considered as one of the possible causes of high-level intestinal obstruction in the immediate postoperative period after partial gastrectomy.

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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

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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

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

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Acknowledgments

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

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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

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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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

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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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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

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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/eid.htm

Patent (list all authors)

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

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

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

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Italics

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

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